INDIAN AGRICULTURAL
RESEARCH INSTITUTE, NEW DELHI.

17082/36

I. A. R. I. 6.
S. C. P—1/8/47-P. J.-17-5-48 2000

# PROCEEDINGS

OF THE

# ROYAL SOCIETY OF LONDON

## SERIES B—BIOLOGICAL SCIENCES

VOL CXIX

LONDON
Printed and published for the Royal Society
By Harrison & Sons, Ltd., 44-47, St. Martin's Lane
Printers in Ordinary to His Majesty

March 2, 1936

LONDON:
HARRISON AND SONS, LTD., PRINTERS IN ORDINARY TO HIS MAJESTY
ST. MARTIN'S LANE

# CONTENTS

## SERIES B VOL CXIX

### No. B 812—December 2, 1935

PAGE

Studies on the Physiology of the Maternal Placenta in the Rat. By H. Selye and T. McKeown. Communicated by J. B. Collip, F.R.S. (Plates 1–6).. 1

Transference of Induced Food-Habit from Parent to Offspring—II. By D. E. Sladden. Communicated by E. W. MacBride, F.R.S................. 31

Studies in Peroxidase Action. I—The Oxidation of Aniline. By P. J. G. Mann and B. C. Saunders. Communicated by Sir F. G. Hopkins, P.R.S. 47

The Effects of X-Rays on Mitosis in the Spermatogonial Divisions of *Locusta migratoria* L. By M. J. D. White. Communicated by J. B. S. Haldane, F.R.S.......................................................... 61

The Principle of Formaldehyde, Alcohol, and Acetone Titrations. (An Addendum.) By G. M. Richardson. Communicated by C. R. Harington, F.R.S.......................................................... 85

The Ultra-Violet Absorption of some Complex Aromatic Hydrocarbons—I. (Abstract.) By W. V. Mayneord and E. M. F. Roe.................... 87

### No. B 813—January 1, 1936

Address of the President, Sir Frederick Gowland Hopkins, O.M., at the Anniversary Meeting, November 30, 1935.............................. 88

Uricase, Amino Acid Oxidase, and Xanthine Oxidase. By D. Keilin, F.R.S., and E. F. Hartree.............................................. 114

Coupled Oxidation of Alcohol. By D. Keilin, F.R.S., and E. F. Hartree...... 141

The Action of Cyanide and Other Respiratory Inhibitors on Xanthine Oxidase. By M. Dixon and D. Keilin, F.R.S................................ 159

Respiratory Variations of the Heart Rate. I—The Reflex Mechanism of the Respiratory Arrhythmia. By G. V. Anrep, F.R.S., W. Pascual, and R. Rössler. (Plates 7 and 8)......................................... 191

Respiratory Variations of the Heart Rate. II—The Central Mechanism of the Respiratory Arrhythmia and the Inter-Relations between the Central and the Reflex Mechanisms. By G. V. Anrep, F.R.S., W. Pascual, and R. Rössler. (Plates 9 and 10)........................................ 218

### No. B 814—February 1, 1936

Studies in Microcataphoresis. I—Technique. By H. C. Brown and J. C. Broom. Communicated by C. M. Wenyon, F.R.S.................... 231

PAGE

Experimental Sodium Chloride Deficiency in Man. By R. A. McCance. Communicated by E. Mellanby, F.R.S. .... 245

Short-Period Fluctuations in the Numbers of Bacterial Cells in Soil. By C. B. Taylor. Communicated by Sir John Russell, F.R.S. .... 269

A Comparison of the Rate of Metabolic Activity in the Solitary and Migratory Phases of *Locusta migratoria*. By C. G. Butler and J. M. Innes. Communicated by A. D. Imms, F.R.S. .... 296

Excitation and Accommodation in Nerve. By A. V. Hill, F.R.S. .... 305

The Measurement of "Accommodation" in Nerve. By D. Y. Solandt. Communicated by A. V. Hill, Sec.R.S. .... 355

Quantitative Spectrographic Analysis of Biological Material. I—A Method for the Determination of Lead in Cerebrospinal Fluid. (Abstract.) By J. S. Foster, F.R.S., G. O. Langstroth, and D. R. McRae .... 380

No. B 815—March 2, 1936

The Effects of Prolonged Muscular Exercise on the Metabolism. By F. C. Courtice and C. G. Douglas, F.R.S. .... 381

The Strength-Duration Relation for Electric Excitation on Medullated Nerve. By A. V. Hill, Sec.R.S. .... 440

Analytic Studies in Plant Respiration. VI—The Relation of the Respiration of Potatoes to the Concentration of Sugars and to the Accumulation of a Depressant at Low Temperatures. Part 3—The Relation of the Respiration to the Concentration of Sucrose. By J. Barker. Communicated by F. F. Blackman, F.R.S. .... 453

The Action of Sodium Nitrate upon the Infection of Lucerne Root-Hairs by Nodule Bacteria. By H. G. Thornton. Communicated by Sir John Russell, F.R.S. (Plates 11, 12) .... 474

Factors Affecting the Formation of Local Lesions by Tobacco Mosaic Virus. By J. Caldwell. Communicated by Sir John Russell, F.R.S. (Plate 13) 493

Researches on Plant Respiration. IV—The Relation between the Respiration in Air and in Nitrogen of certain Seeds during Germination. (*b*) Seeds in which Carbohydrates Constitute the Chief Food Reserve. By W. Leach. Communicated by W. Stiles, F.R.S. .... 507

Index .... 522

671 . 013 . 85

# Studies on the Physiology of the Maternal Placenta in the Rat

By H. SELYE, Assistant Professor of Biochemistry, and T. MCKEOWN,
National Research Council Scholar

(From the Department of Biochemistry, McGill University, Montreal, Canada)

(*Communicated by J. B. Collip, F.R.S.—Received May* 13, 1935)

[PLATES 1-6]

Although a great many investigators have made adequate studies on the maternal placenta from the morphogenetic point of view, little attention has so far been given to the humoral conditions which make the formation of the placenta materna possible. Experimental investigations of the problems of placental physiology have as yet been few, and leave unanswered many important questions. Which are the factors determining the life span of the placenta, and what is responsible for its ageing? In what way are the pregnancy changes influenced by the three structures —maternal placenta, foetal placenta, and embryo—present during gestation, and what is the role of each ?

Recent advances in the physiology of reproduction have made it possible to attempt the elucidation of these problems under unusually favourable conditions. Of great importance was the observation of Leo Loeb that the maternal placenta could be produced experimentally in the rodent. Furthermore, we have reported experiments which showed that the embryos could be removed from the pregnant rat and mouse without interfering with the development of either the foetal or the maternal placenta. These facts, together with the important progress of the past few years in the physiology of the female sex hormones in general, have formed the basis for our present studies, and have enabled us to elucidate some of the problems indicated above.

Many of the observations on the physiology of the maternal placenta have been presented in the literature as incidental findings, rather than as the product of a direct investigation. It is obviously of importance that these findings should be correlated, and since no attempt has previously been made to do this, we have thought it worth while to review the literature as completely as possible.

We have divided our material into three main sections. The first deals with deciduoma formation—that is, a reaction of the uterine stroma dependent on the presence of corpus luteum hormone. The second comprises the experiments on the formation of the endometrial mole—a reaction of the uterine stroma dependent on the presence of oestrin. In the third part, we describe the insertion gland—a structure situated in the muscle and mesometrium at the site of the placental insertion, and dependent for its formation on the presence of corpus luteum hormone.

## I—The Deciduoma

In 1907, Leo Loeb first described the production of the deciduoma in the guinea-pig. He was able to show that the maternal placenta, normally produced under the influence of the fertilized ovum fixing itself in the mucosa of the uterus, could also be formed under experimental conditions. On the basis of his observations he concluded that " the experimental production of the maternal placenta depends primarily on three factors: first, on a chemical sensitizing of the uterine mucosa; second, on an additional mechanical stimulus applied to the uterus; and third, on a certain condition of the body fluids of the animal."

There appears to be little doubt that an important essential to the appearance of the reaction is the presence of functional corpora lutea in the ovary at the time when the uterine irritation is performed. This is independent of the species, for it has been reported in the rat, mouse, rabbit, guinea-pig, dog, and monkey, during those periods when active corpora lutea are present. The latter may occur, however, under many conditions, such as pregnancy, the pseudo-pregnancy produced by sterile mating, lactation, the diloestrus consequent to the application of the stimulus of suckling, as well as under treatment with gonad-stimulating extracts.

Loeb's observations were first extended by Frank (1911) who produced deciduomata in the rat, guinea-pig, and rabbit. The work was soon confirmed: on the rat by Gasbarini (1911); on the rabbit by Hammond (1917), Nielsen (1921), Biedl, Peters, and Hofstätter (1921); and on the guinea-pig by Gasbarini (1911), Desclin and Brouha (1931). Furthermore, it has been extended to the mouse (Parkes, 1929); to the dog (Krainz, 1914); and to the monkey (Engle and Shelesnyak, unpublished data referred to by Shelesnyak, 1933, *b*). It is reported by Aschner (1918) that he obtained negative results in the guinea-pig; however, as Biedl *et alii* point out, he attempted to produce it at the wrong period.

It is generally believed that the reaction can be brought about by corpora lutea only from the third to the eighth days following the ovulation which produced them, irrespective of the conditions under which the persistent corpora have been formed. (To this, however, there is a notable exception—the condition of lactation, which we shall discuss later.) Loeb (1907), and later Frank (1911), stated that the time when deciduomata may be produced is limited. Nielsen (1921) observes that the reaction follows uterine trauma performed during the third to seventh-day interval following copulation in the pseudo-pregnant rabbit. In the guinea-pig Brouha (1932) has shown that the response is positive only from the fourth to the seventh day after ovulation. The results in experimental production of corpora lutea by administration of anterior pituitary extracts, in pregnancy and in the castrate with ovarian hormones, are in good agreement.

It is possible to produce deciduomata in the guinea-pig in the course of the normal cycle of 16 days, but not in the rat (Long and Evans, 1922) and mouse (Parkes, 1929), animals in which only some 4 days normally separate the appearance of oestrus, and in which the life of the corpus luteum is a short one. In the rabbit, as is well known, ovulation—and hence the formation of corpora lutea—is only brought about by copulation.

### *General Morphology of the Deciduoma*

Before discussing the histological characteristics of deciduomata we should like to call attention to a few morphological characteristics of the rat uterus, which we believe to be essential for the comprehension of the reactions involved in deciduoma formation. The muscular wall of the organ consists of two layers, an external longitudinal and an internal circular layer. The external layer not only surrounds the uterus proper but extends far into the mesometrium, the vessels of which thus come to lie between two lamellae of smooth muscle fibres, which are the direct continuation of the external muscle coat of the uterus. Between the vessels there are more or less irregular strands of smooth muscle fibres which connect the two lamellae of the mesometrial prolongation of the longitudinal coat. The mesometrial blood vessels form numerous ramifications as soon as they reach the uterus, thus forming a triangular area (on transverse sections) limited on two sides by the mesometrial extension of the longitudinal, and on the third side by the mesometrial, surface of the circular muscle coat, fig. 1, Plate 1. As will be seen later, very significant changes occur in this area during pregnancy and during deciduoma formation. We may now describe the conditions obtained during lactation—a state in which deciduomata are very readily formed. Inside the circular muscle coat is the stroma of the mucosa, very rich in cells and relatively poor in fibres during lactation. The nuclei of its cells are round and larger than usual. The epithelium lining the uterine cavity is cuboidal or short cylindrical and its surface is free of cilia. A very characteristic feature of the epithelial cells during lactation is the deposition of lipoid granules in the basal portion of their cytoplasm, fig. 2, Plate 1. This condition of the mucosa, which we designated as the "first progestational reaction" (Selye, Collip, and Thomson, 1935) is maintained during the entire lactation dioestrus. Deciduomata may only be produced while the stroma mucosae is in this condition. If the mucosa is irritated by traumatization in this stage its first reaction to the stimulus is a proliferation of decidual cells in the subepithelial region. These cells grow and multiply very rapidly and form a tumour which occupies the entire cavity of the uterus and distends its walls, fig. 3, Plate 1. Soon two distinct regions may be distinguished in these deciduomata. One consists of small vacuolized cells and is invariably situated on the mesometrial side; the cells of the other one on the antimesometrial side are much larger and have a homogeneous, slightly basophilic cytoplasm with no visible cellular inclusions, figs. 4 and 5, Plate 1. Sections stained with Best's carmine show that the vacuoles in the small cells are originally filled with glycogen. These small cells with their glycogen granules closely resemble the cells of the maternal placenta found in the corresponding region (*i.e.*, in the vicinity of the mesometrium just below the placenta) in pregnancy. It is interesting to note that these glycogen storing cells will always form on the mesometrial side of the symmetrical uterine lumen quite irrespective of the site of the traumatism which produced the deciduoma. It seems, therefore, that the reason for the invariable formation of the rodent placenta on the mesometrial side is in the uterus itself and not in the ovum. The deciduoma usually reaches its maximum development between the fifth and sixth days after the productive stimulus has been applied. At this period the central portion of the deciduoma undergoes necrosis and gradually disappears; the glycogen storing cells persist much longer. At about the fifth or sixth day—that is at the time when the central portion of the tumour degenerates—there is a marked reaction in the insertion region. The endothelial cells of the vessels become much larger than usual and the connective tissue between the blood vessels of this area becomes very loose. Both the decidual cells in the endometrium and the adventitious cells of the blood vessels proliferate very actively at this period, and show numerous mitotic figures. At approximately the fifth day the proliferating decidual cells invade the mesometrial portion of the circular muscle coat, thus establishing a direct connexion between the insertion region and the mucosa, fig. 6, Plate 1. They are more or less round, dedifferentiated cells, and they resemble closely those elements which at this same period form from the cells of the blood vessel walls. We believe that similar elements are also formed from the smooth muscle cell strands connecting the two lamellae of the mesometrial continuation of the longitudinal muscle coat in the insertion area. It seems that it is not any particular mother cell that gives origin to these elements, but that almost any cell in this region may do so under the influence of some local stimulus, by a dedifferentiation into a cell type resembling at this stage an immature fibroblast. Somewhat later, between the fifth and sixth days, these dedifferentiated cells enlarge and chromophil granules appear within them. Most of these granules are acidophilic, but some of them are distinctly basophilic. The former stain with acid fuchsin or eosin, while the latter can be stained particularly well with anilin blue. Both types of granules may be found in one and the same cell. The following staining technique was found to give most satisfactory results.

1—Fix in Haidenhain's "Susa" mixture; embed in paraffin; cut sections, not thicker than 3 microns.
2—Stain in Ehrlich's haematoxylin; differentiate in acid alcohol; wash in running water for 2 hours, then 1 hour in distilled water, changing every 15 minutes.
3—Stain 3 minutes in 5% aqueous solution of acid fuchsin; then rinse in distilled water for 5 seconds.
4—Stain for 3 minutes in 2% solution of orange G in 1% phosphomolybdic acid; then rinse in distilled water for 5 seconds.
5—Stain for 2 minutes in 1% aqueous solution of aniline blue; then rinse in distilled water; dehdyrate in ascending concentrations of alcohol.
6—Clear in xylol and mount in Canada balsam.

With this method the nuclei stain dark blue (haematoxylin), the basophilic granules lighter blue (anilin blue), and the acidophilic granules red (fuschsin). We should like to mention at this point that this same method is also constantly used in our laboratory for the staining of pituitary glands.

At the time when the intraumerine part of the tumour—the characteristic decidual tissue—degenerates, the granulated cells in the insertion area reach their full development. At this period a structure is formed in the mesometrial wall of the uterus and the adjacent mesometrium identical with the " metrial gland " which forms during pregnancy and which is described in the third part of this communication, fig. 6, Plate 1.

### *Deciduoma Formation during Lactation*

Corner and Warren (1919) were the first to observe that the corpora lutea of lactation could sensitize the uterus to the formation of deciduomata. They worked on the rat, and performed the irritation on the seventy-day post partum. This was later repeated by Long and Evans (1922) on the rat, and by Parkes (1929) on the mouse. Parkes points out that although the reaction could occur in the lactating mouse, it was not so marked as in the pseudo-pregnant animal. Long and Evans on the rat were able to get a consistent response after the 4th day and until the 10th day, with occurrence even as late as the 16th day post partum. Leonard and Meyer (unpublished data) have produced deciduomata in lactating rats as late as 23 days post partum.

We produced irritation of the endometrium, by the usual technique of drawing a thread through the uterine wall, in a group of 16 rats, one on each day from the day of delivery to the 16th day post partum, the uterus being examined in all instances 4 days after operation. The results were negative in the first 4 days and in the last 3; in the remaining cases the animals had well-developed deciduomata. The response was positive then in 100% of cases between the 5th and 13th days.

In an attempt to see whether the response could occur before the 4th day, we placed the threads in eight animals, two a day, during the first 4 days post partum. The reaction was observed in one of the animals operated on the first day, and in one of those operated on the fourth; all others were negative.

Since the development and maintenance of the deciduomata are dependent upon the action of the corpora lutea, it seemed reasonable to expect that the existence of the decidual tissue would be a function of the life of the corpora themselves. In studying this question, we placed threads in a group of 12 animals on the 5th day and examined them, two a day, between the 11th and 16th days post partum. We saw deciduomata well developed at the 11th and 12th days, involuting at the 13th and 14th days and absent (except in one instance) on the 15th and 16th days. It appears that the decidual tissue involutes at a definite time, even though the corpora be still present, showing no signs of involution, and apparently active, since dioestrus continues.

In the lactating mouse we were unable to obtain deciduomatous growths as frequently as Parkes (1929). The uterine trauma was performed in

36 animals in various stages of the lactation period. Only in three instances were deciduomata seen on examination at the usual time.

In a recent communication, we have shown that the stimulus of suckling produced in the normal rat and mouse a dioestrus characterized by the presence of functional corpora lutea (Selye and McKeown, 1934). We observed that it was possible during this condition to produce deciduomata by irritation of the uterine mucosa.

### *Deciduoma Formation during Pregnancy*

Loeb's early studies (1907) were made upon the pregnant guinea-pig, and he was able to establish the fact that for a limited time in the pregnant animal uterine irritation led to the production of deciduomata. It is only during recent years that any further investigation has been made of the decidual response in pregnancy. Courrier and Kehl (1930) were able to produce deciduomata in the sterilized horn of the uterus of the pregnant rabbit when the trauma was performed on the 5th, 6th, and 8th days of gestation, but not if the animals were operated on the 12th, 16th, or 20th days. This was confirmed by Allen (1931) who obtained positive results on the 4th, 5th, 6th, and sometimes the 7th days, but never after that time. Deciduoma production between the gestation sacs in the pregnant horn has not been attempted at corresponding stages of gestation, and therefore one feels that the results of this experiment do not necessarily prove an abnormal behaviour of the sterilized horn as such, but might be explained equally well by the assumption of some factor inhibiting deciduoma formation during the later stages of pregnancy.

In order to obtain more definite information on this subject, we performed the following experiments: a group of 15 rats was sterilized in one horn by the excision of a small portion of the tube near the ovary and allowed to become pregnant. Threads were placed in the sterile horn, and in some instances also in the pregnant horn between the foetuses, in animals at various stages of pregnancy, ranging from the 9th to the 15th days.

Examination 5 days after operation revealed the fact that in all the animals the reaction had been negative.

We placed threads, on the 5th day of pregnancy, in the sterile horn of four animals, prepared in the same manner as those of the previous group and with pregnancy indicated by examination of the vaginal smear. Four days later these animals showed well-developed deciduomata. That the latter could not be maintained in the later stages was also noted, *for 4 days later, i.e., 8 days from the date of placement of the thread, they had disappeared.*

*It seems, then, that while the decidual response may be elicited in the early stages of pregnancy it fails in the later stages; and further, that the time at which the change in the uterine reaction occurs is approximately* coincident with nidation and with the appearance of the placenta. The importance of the placenta is emphasized by experiments recently reported from this laboratory, in which it was observed that the placenta could survive in the absence of ovarian and foetal tissue (Selye, Collip, and Thomson, 1935). We designed the following experiment, in an attempt to demonstrate a specific placental inhibition to the formation of deciduomata.

Seventeen female rats sterilized in one horn were allowed to become pregnant. On the 7th day threads were placed in the sterile horns of five animals, and in all animals Caesarean section was also performed. The horns of the 12 animals previously untouched were traumatized 2 days later, *i.e.*, on the 9th day. Vaginal smears were taken, and the uteri examined 2 to 4 days later.

In no animal in which the thread had been placed at the time of Caesarean section was the reaction positive. Of the 12 remaining animals, 8 examined within 4 days of traumatization showed well-developed deciduomata. In none of these animals had vaginal oestrus appeared. The other four animals were examined on the 4th day, immediately after the appearance of oestrus. In these instances no deciduomata were present. This would indicate that inhibitory factors may arise which are due to the presence of the placenta, and which make it impossible to obtain the decidual response. If, however, we traumatize some time after the placenta has been removed, then again the reaction is positive.

The possibility arises that the decidual tissue could exhibit an inhibition analogous to that of the placenta. We attempted to demonstrate this in the lactating animal. For this we took 14 animals, normally lactating. On the 5th day post partum we placed threads in the right horns of the uteri of 11 of them, being careful that the left horn should not be touched. Seven days later these were all re-opened, well-developed deciduomata well noted in one horn and the other was found to be stimulated in the usual manner. Threads were also placed at this time, *i.e.*, 12th day post partum, in the three control animals. All were killed in 5 days.

It was observed that all the controls showed well-developed deciduomata. Of the 11 experimental animals, however, only one showed a comparable growth in the left horn, five had very small deciduomata, while the remaining five were negative.

Our findings indicate, then, that inhibitory factors may arise to the formation of deciduomata: in the placenta, in all probability; in pregnancy, and in decidual tissue previously present during lactation. The nature of this influence we shall not discuss at this point, but will consider it in connexion with our experiments on the effect of oestrin on the reaction.

The data discussed in reference to the decidual response over the course of pregnancy tend to support the conception of a difference in the mechanism involved in the control in the utero-ovarian interrelations in the earlier and later parts of pregnancy. This is also the implication of experiments to which reference has already been made, in which it was seen that only in the first stage is the ovary essential to the life of the placenta (Selye, Collip, and Thomson, 1935). That the ovary must be present in the rat, at least until nidation has taken place, seems unquestionable, however, since castration before the 8th day will inevitably interfere.

It has been known, since the reports of Ancel and Bouin (1911), that sterile mating leads to a condition closely resembling pregnancy, to which the name " pseudo-pregnancy " is given. Like pregnancy, it is characterized by the absence of oestrus, presence of large functional corpora lutea, development of the mammary gland, and progestational changes in the uterine mucosa, with susceptibility to deciduomata formation. This condition continues for about 13 days in the rat and mouse, at which time the corpora undergo involution and normal cycles become established.

One may wonder, then, whether the changes which occur in the early part of pregnancy are not consequent to the nervous stimulation, in spite of the fact that the mating was fertile, and that the development of foetus and placenta resulted. If so, Caesarean section ought not to lead to the involution of the corpora lutea, nor to the return of oestrus until such time as the response to the nervous stimulus had been exhausted, *i.e.*, at the usual time of termination of pseudo-pregnancy.

This we have studied in the mouse and in the rat. It was observed that in five mice vaginal oestrus did not reappear in any animal within a week as a result of Caesarean section on the 7th day of pregnancy, whereas in five control animals, operated on between the 12th and 18th days, it reappeared within 52 hours. This was confirmed in six rats in early pregnancy (7th day) with 10 controls in late pregnancy.

### *Effect of Ovariectomy on the Deciduoma*

It was observed by Loeb (1908) that the presence of the ovaries was essential to the production of deciduomata in the guinea-pig. Corner and Warren (1919) noted that if the ovaries were removed from the rat at the time when trauma was performed the reaction was negative. This was confirmed by Long and Evans (1922). In the pseudo-pregnant guinea-pig, however, the results are contradictory. Weichert (1928) obtained no response if the animals were castrated; Courrier (1930) states that under such conditions deciduomata are produced but may only be seen microscopically; while Raynaud (1934) found that the absence of the ovaries did not interfere with the reaction.

Our own observations on the rat support those of Loeb, for we were never able to elicit the response in the castrated animal. We castrated two lactating rats on the 7th day post partum, at the same time placing threads in the uteri in the usual manner. Examination 5 days later showed that the reaction had not occurred. This experiment is well controlled by our observations on normal lactating animals, in which we have had positive results in all animals at this period.

Furthermore, it was observed with three lactating rats in which deciduomata were well developed that removal of the ovaries led to immediate involution of the tissue. The ovaries were removed on the 10th day in animals in which deciduomata were present as a consequence of uterine trauma at the 6th day. In all three instances the tissue had involuted at the 13th day. Unlike the placenta, the deciduoma is unable to maintain itself in the absence of ovarian influence.

### *Deciduoma Formation under the Influence of Hormone Preparations*

It is to-day well established that in the normal animal the administration of anterior pituitary gonad-stimulating preparations can lead to a condition in which the decidual response may be elicited. This has been reported in the adult rat after uterine irritation by Teel (1926) and Bouha (1928), while Innes and Bellerby (1929) have noted the spontaneous appearance of the reaction. Shelesnyak produced deciduomata in the immature rat after treatment with pituitary implants followed with anterior pituitary extracts (1931), after administration of oestrin and anterior pituitary extracts (1933, *a*), and in about 75% of cases with pregnancy-urine preparations (1933, *b*). There were some responses to anterior pituitary extracts alone. Evans and Simpson (1929) reported the formation of deciduoma after administration of anterior pituitary " growth hormone " extracts to adult virgin rats, and also after treatment with the anterior pituitary sex factor if the latter is not given so as to lead to the production of too much folliculin. Friedman (1932) notes *that the corpora lutea produced in the rabbit with anterior pituitary extracts can lead to the decidual response.*

Attempts have also been made to prepare the uterus with ovarian hormones in the absence of the ovary. Weichert (1928) states that although positive results could be obtained in the normal rat with corpus luteum hormone, provided that the irritation was performed at the 3rd day after the last oestrus, in the castrate the action could occur only in those animals in which oestrin had first been present. This was confirmed by Nelson and Pfiffner (1930) in the adult rat and by Shelesnyak (1933, *c*) in the immature animal. In the guinea-pig the findings are in good agreement. In this species Weichert (1928) showed that although corpus luteum treatment alone could sensitize the uterus in the absence of the ovaries, it could only do so for a limited period after oestrus (until the 10th day). Goldstein and Tatelbaum (1930) also reported positive results in the castrate virgin guinea-pig treated with a corpus luteum preparation.

It seems to be quite definite, then, that both follicular and corpus luteum factors are necessary for the production of the decidual response. This co-ordinate action has been observed by Hisaw and Leonard (1930) and by Leonard, Hisaw, and Fevold (1932), who showed that the progestational changes in the uterus are produced by corpus luteum hormone and folliculin in combination, although neither alone is effective in the castrate. Brouha (1928) has been led to suggest that the eventual failure of the uterus to respond to irritation during corpus luteum action is to be explained by loss of the sensitiveness brought about by the last influence of oestrin.

In apparent contradiction of these statements is the report of Loeb and Kountz (1928) that injections of oestrin do not prepare the uterine mucosa to deciduoma formation, but rather tend to inhibit the decidual changes produced by corpus luteum hormone. There appears to be little doubt, however, that oestrin can inhibit the decidual reaction. It is known from the work of Allen (1932) and others that oestrin can inhibit the action of corpus luteum on the uterus of the castrate animal, and in large doses can prevent the proliferation of pregnancy. Courrier (1930) and Brouha (1932) have shown that the follicular hormone can inhibit the formation of deciduomata in the pseudo-pregnant guinea-pig, and Courrier (1930) has also demonstrated this on the lactating animal.

Parkes (1929) and later Robson (1935) have reported that oestrin itself is inhibited in the lactating mouse.

We have been interested in the investigation of this inhibition and have made a study of the problem in the lactating rat. In our preliminary experiments we took 12 lactating rats, 6 of which were castrated on the day after delivery, and all of which were given 15 $\gamma$ of oestrone daily. The same dose of oestrin was also administered to 6 normal castrated control animals. The vaginal smears showed that while the normal controls were all brought into continuous oestrus under this treatment there was no appearance of oestrus in the lactating non-castrate animals. More remarkable, however, was the fact that an inhibition was also observed in the lactating castrates, in which oestrus was indicated only at irregular intervals of about 5 days' duration. On increasing the dose, we saw that continuous oestrus could be obtained in the lactating ovariectomized animal, at a level still completely inhibited in the non-castrate (25–100 $\gamma$ per day).

In a study of the inhibitory influence of oestrin on the formation of deciduomata, we gave 20, 30, 40, and 100 $\gamma$ daily to each of four animals from the 4th day post partum, placing threads in the uteri on the 7th day, and examining the uteri on the 12th day. In three animals the reaction was negative; the animal receiving 30 $\gamma$ had a very small deciduoma. The histological study showed that in all the animals the uteri were of the type typical of the second stage of pregnancy, brought about obviously by the oestrin influence, fig. 7, Plate 1.

These experiments suggested the possibility that our failure to obtain the deciduoma reaction in one horn of the lactating animal in those cases in which deciduomata were already present in the other horn might be explained by the fact that the uterus was, at the time of irritation, in the condition typical of the second part of pregnancy. This was found to be so, for in six lactating animals in which deciduomata were well developed in the traumatized horns it was observed that the other horns showed the progestational development that may otherwise be brought about by administration of oestrin in the presence of an active corpus luteum. Yet the degree of the changes in the mucosa in these instances indicates that the quantity of oestrin liberated in the presence of a deciduoma must have been incomparably smaller than that present during the second half of pregnancy. The most sensitive index of oestrin action in the presence of an active corpus luteum seems to be the disappearance of the lipoid granules in the basal portion of the epithelial cells lining the lumen of the uterus. These granules are invariably present at a time when deciduomata may be produced, that is, during the first part of pregnancy or of pseudo-pregnancy, and during lactation. They vanish after the administration of oestrin, and are absent in all those conditions in which we suspect oestrin production in the presence of a corpus luteum. This is so in late pregnancy and in the non-traumatized horn of the uterus of animals bearing a deciduoma on the other side.

It appears reasonable to suppose, then, that the decidual response occurs when traumatization of the uterus is performed at a time when the mucosa is in the first progestational stage; so one obtains it in the early part of pregnancy and through all the period of lactation. But the appearance of the second stage development, normally occurring in the latter half of pregnancy and induced in lactation either by oestrin or by the presence of decidual tissue, makes it impossible to elicit the response.

### *Deciduoma Formation after Unspecific Inhibition of Follicle Maturation*

There remains for discussion the occurrence of the reaction during certain unusual conditions somewhat removed in their general character from those in which our conception of the nature of the response offers an obvious explanation. One of the most striking instances results from the administration of desiccated thyroid. Numerous investigators have seen that this can lead to the suppression of the cycle (Cameron and Amies, 1926; Reiss and Perèny, 1928) and to prolonged corpus luteum activity (Weichert, 1930; Weichert and Boyd, 1933; Van Horn, 1933; and Campbell, Wolfe, and Phelps, 1934). In this condition Weichert and Boyd (1933) were able to elicit the decidual response by uterine trauma, and they suggest the possibility of increased pituitary stimulation of the ovaries under the treatment. That thyroid feeding does increase the gonad-stimulating power of the anterior pituitary has been shown by Evans and Simpson (1930) and by Van Horn (1933).

It is, perhaps, more remarkable that deciduomata may be formed as a consequence of vitamin deficiency. Bishop and Morgan (1928) observed spontaneous deciduomata in rats showing vitamin A deficiency, also in rats of vitamin E deficient diet if also low in vitamin A. Evans (1928) reported that about 60% of rats mated with vasectomized males showed spontaneous deciduomata if on a vitamin E deficient diet as against 4% if not.

An explanation suggests itself, however, which might conceivably offer a common basis for these apparently unrelated instances. In each of the conditions referred to above the animals were exposed to severe treatment and were losing weight. If one supposes that the follicular or oestrus phase of ovarian activity is more sensitive to such stress than is the luteal phase, then conceivably one might interfere with it sufficiently to allow the luteal phase to manifest itself longer than usual.

In order to test this hypothesis, we have repeated the observations on thyroid fed animals and have studied other conditions in which the occurrence of the decidual reaction might only reasonably be explained by the theory just elaborated.

In our repetition of Weichert's work, we used 12 adult rats, to each of which we gave 0·5 gm of desiccated thyroid daily. A condition of dioestrus appeared soon after the beginning of the treatment, and uterine trauma was effected in the usual manner. This led to the formation of deciduomata if performed at about the 6th day of dioestrus, but never if done in late stages. In no animal did we see mammary gland development. The maturation of follicles was obviously inhibited in the ovaries of these animals, but, unlike other pseudo-pregnant conditions, there were no persistent corpora lutea. The corpus luteum of the last cycle showed marked signs of involution on the 14th day of dioestrus, and the ability to produce deciduomata in these cases must therefore be ascribed to the absence of the next follicular phase, with its concomitant oestrin production rather than to the persistence of the last corpus luteum.

It is generally agreed that bilateral adrenalectomy leads, in the rat, to a cessation of the cycle, the dioestrus in most instances setting in soon after the operation. This we have previously confirmed on our own colony, and we offered evidence in support of the belief that the dioestrus was a result of the cachexia rather than of the lack of any specific adrenal influence (Kutz, McKeown, and Selye, 1934). So it occurred to us that in the adrenalectomized animal one might be able to demonstrate a luteal effect, the oestrin phase being obviously inhibited.

For this work we used 24 female rats from which the adrenal glands had been removed, and we placed threads in the uteri on the 4th or 5th days of the dioestrus. Examination was made 5 days later of those animals surviving at the end of the period.

In some animals deciduomata were seen macroscopically; in most the uterine reaction was confirmed by histological section. The ovaries contained functional corpora in various stages of involution and no maturing follicles. The mammary glands showed the beginning of acinus formation.

As a final test of our hypothesis, we made use of the severe stress resulting from starvation. Twelve female rats were given insufficient food for a period of several days. A few days after the beginning of deprivation, dioestrus set in, and oestrus did not appear so long as the diet was markedly inadequate. Threads were placed in the uteri on the 5th day of dioestrus, as in our previous experiments.

Examination of these animals 5 days later failed to reveal the presence of any uterine reaction. However, histological studies of the uteri at the points of trauma indicated that a slight decidual response had occurred, although it had not been seen macroscopically.

### *Discussion*

The maternal placenta may be produced experimentally under rather varied conditions. These include a number of instances—such as the early stages of pregnancy, the lactation dioestrus, the pseudo-pregnancy produced by sterile mating, the dioestrus induced by suckling, the dioestrus after treatment with anterior pituitary gonadotropic hormone—characterized by the presence of functional corpora lutea, and so, easily explained as consequences of the activity of the hormone. However, the observations of deciduoma formation during thyroid feeding and on vitamin deficiencies presented some difficulty in interpretation. We have suggested the possibility that in these animals the severe stress could inhibit follicular maturation in the ovary, and so the presence of corpora lutea might be manifested. For this we have found support in the production of the decidual response in animals after adrenalectomy, and in animals during periods of fasting.

It is generally agreed that the deciduoma may be formed only for a limited period after the oestrus which produced the functional corpora lutea. This has been explained previously by the suggestion that the preparatory action of oestrin on the uterine mucosa is essential to the reaction, and that in later periods results are negative because such influence has been lost. So, supposedly, deciduomata are not formed in the later stages of pregnancy, or of pseudo-pregnancy, in spite of the presence of unquestionably functional corpora lutea.

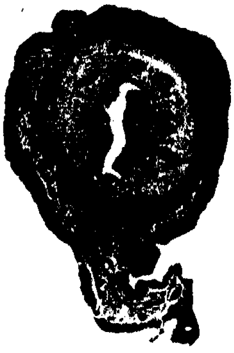
There is a notable exception in the condition of lactation, throughout which one may obtain positive results (even as late as 16 days post partum in the rat) until just shortly before the appearance of oestrus. So we thought it possible that the negative response in the other instances might be explained by the introduction of some inhibitory factor rather than by the loss of sensitivity of the uterus in the interval since the last oestrus. For this we have found support in our demonstration of such an inhibition arising from the presence of the placenta in pregnancy, as well as by a similar inhibition which may be exhibited by the presence of the deciduoma itself in lactation.

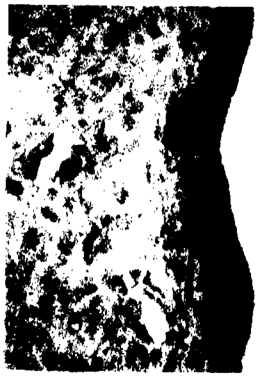
This inhibitory factor we believe to be oestrin. It is well known that at any time oestrin can inhibit the formation of deciduomata. The time at which it becomes impossible to obtain the response in pregnancy is approximately coincident with that at which the uterine mucosa is converted to the second progestational stage—an effect which we believe to be related to the presence of oestrin, especially since it may be elicited by administering oestrin during the period of activity of the corpora lutea of lactation. We have also seen that in lactation, in the presence of the decidual tissue, the uterus—which normally remains in the first progestational state—is converted to the second. So, too, the limited period of response after treatment with gonadotropic hormone may be understood to result from the production of oestrin from ovarian sources. In other cases, however, for example, when the deciduoma reaction eventually fails during the dioestrus produced by thyroid feeding, it seems more probable that luteal activity has declined below the threshold without being eclipsed by oestrin secretion.

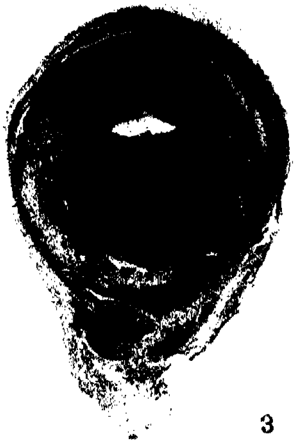
## II—THE ENDOMETRIAL MOLE

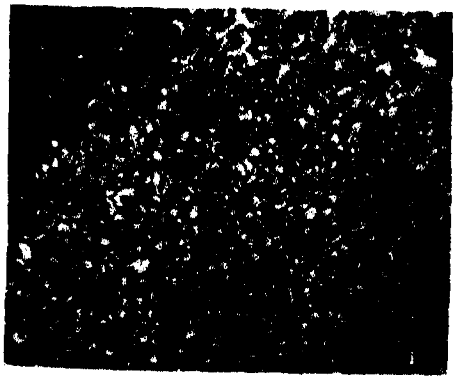
The traumatization of the uterine endometrium with a needle leads to no specific reaction in the oestrin-treated ovariectomized rat. It would seem, therefore, that unlike the corpus luteum hormone oestrin does not sensitize the uterus to trauma. We found, however, that if the uterus of oestrin-treated ovariectomized animals is slit open the endometrium shows a very marked and rather curious reaction which we shall briefly describe.

Twelve ovariectomized rats were treated with daily subcutaneous injections of 30 $\gamma$ of oestrone dissolved in oil. Two days after treatment was initiated, one horn of the uterus was slit open. Biopsy specimens taken 3 days after the operation showed marked oedema of the mucosa, to such a degree that a large tumour-like mass is formed. The water accumulation between the connective tissue fibres was very pronounced at this period. Since slitting the uterus never caused any similar change in untreated ovariectomized or untreated normal females, we had to conclude that this type of reaction to the trauma was due to a specific influence of oestrin on the endometrium. Histologically the stroma of this tumour resembles strikingly the connective tissue of a hydatidiform mole. The surface of the tumour is lined by the epithelium of the uterus, figs. 8 and 9, Plate 2. Since the tumour forms from the stroma of the endometrium and structurally resembles a hydatidiform mole we shall refer to it in our discussion as the " endometrial mole " (Selye, Harlow, and McKeown, 1935). It is to be remembered that there is no intimate relationship, other than the morphological similarity, between this structure and the hydatidiform mole, since the latter is derived from the foetal part of the placenta.

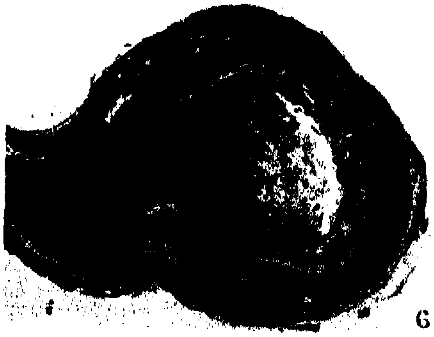

1

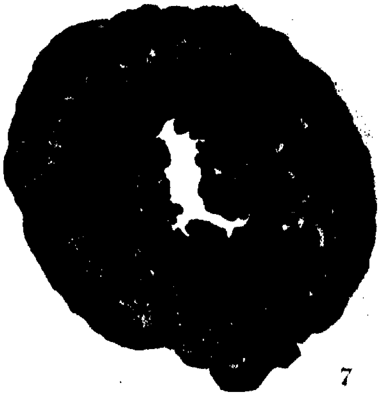

2

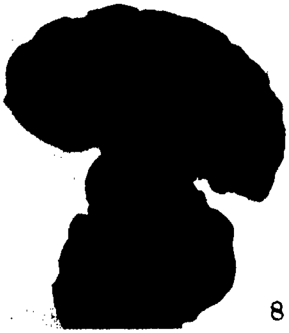

3

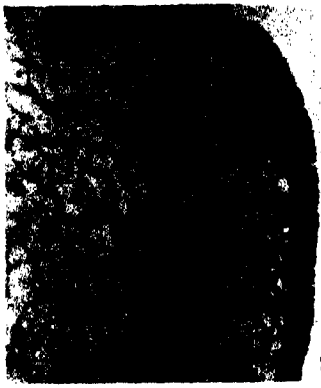


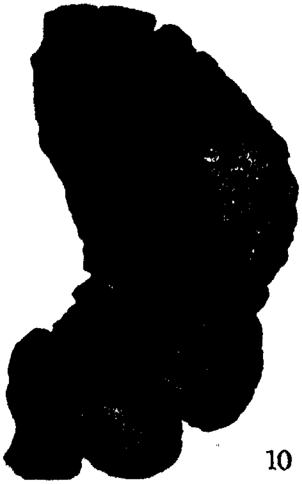
5


6

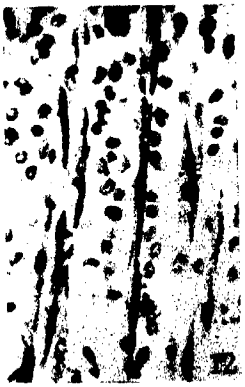

7

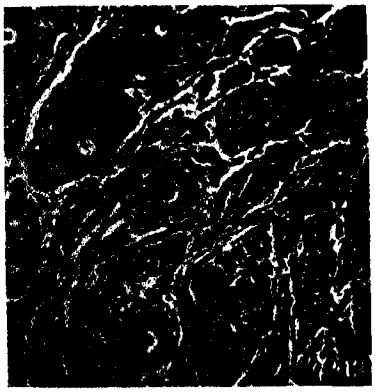

8

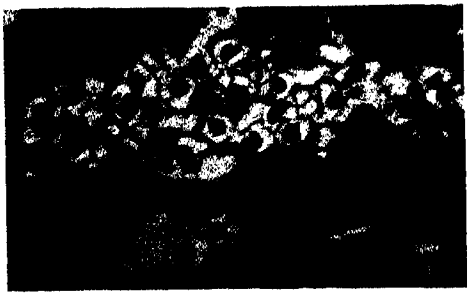

9

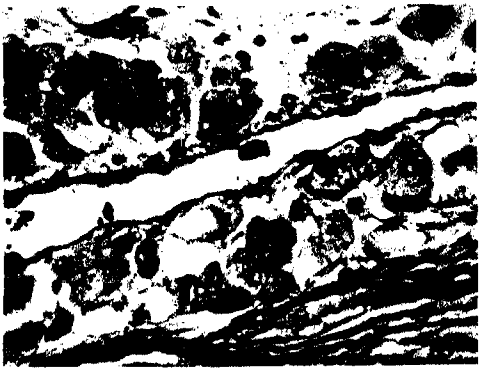

10

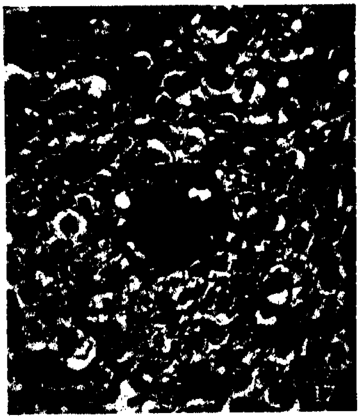
11

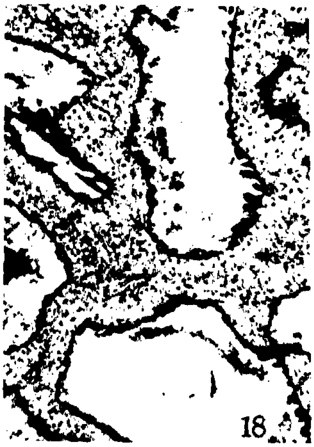

12

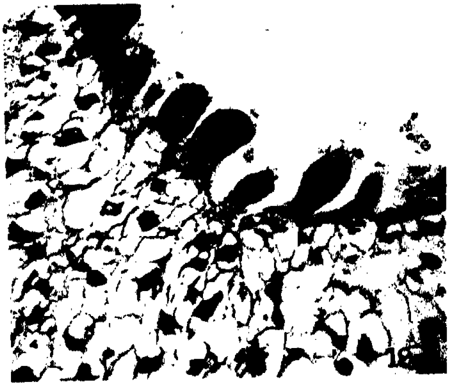


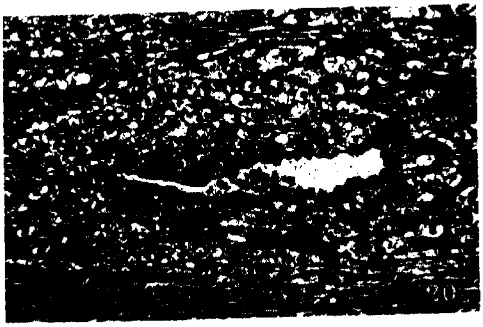





17


18

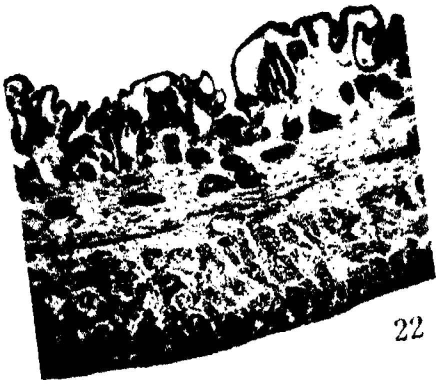

19

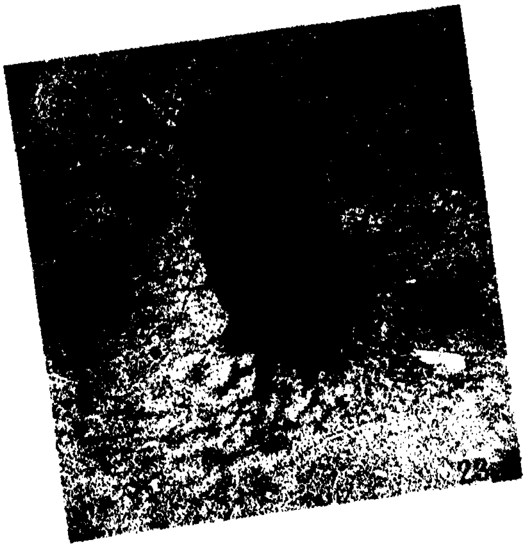

20

Selye and McKeown

*Proc. Roy. Soc., B, vol. 119, Plate 4*

21

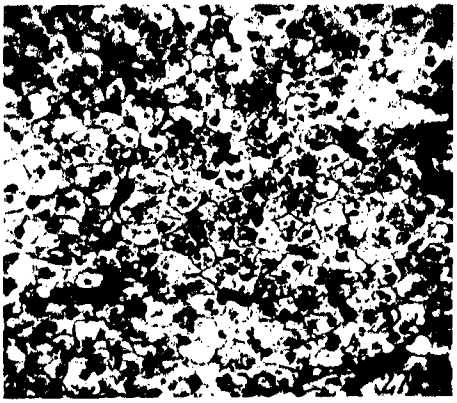

22

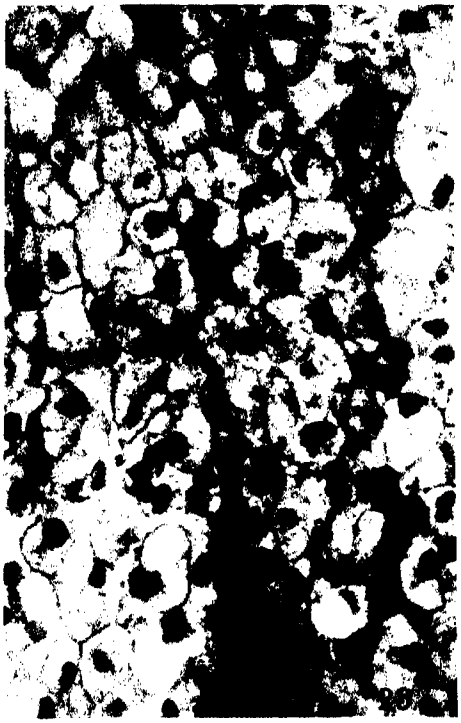

23

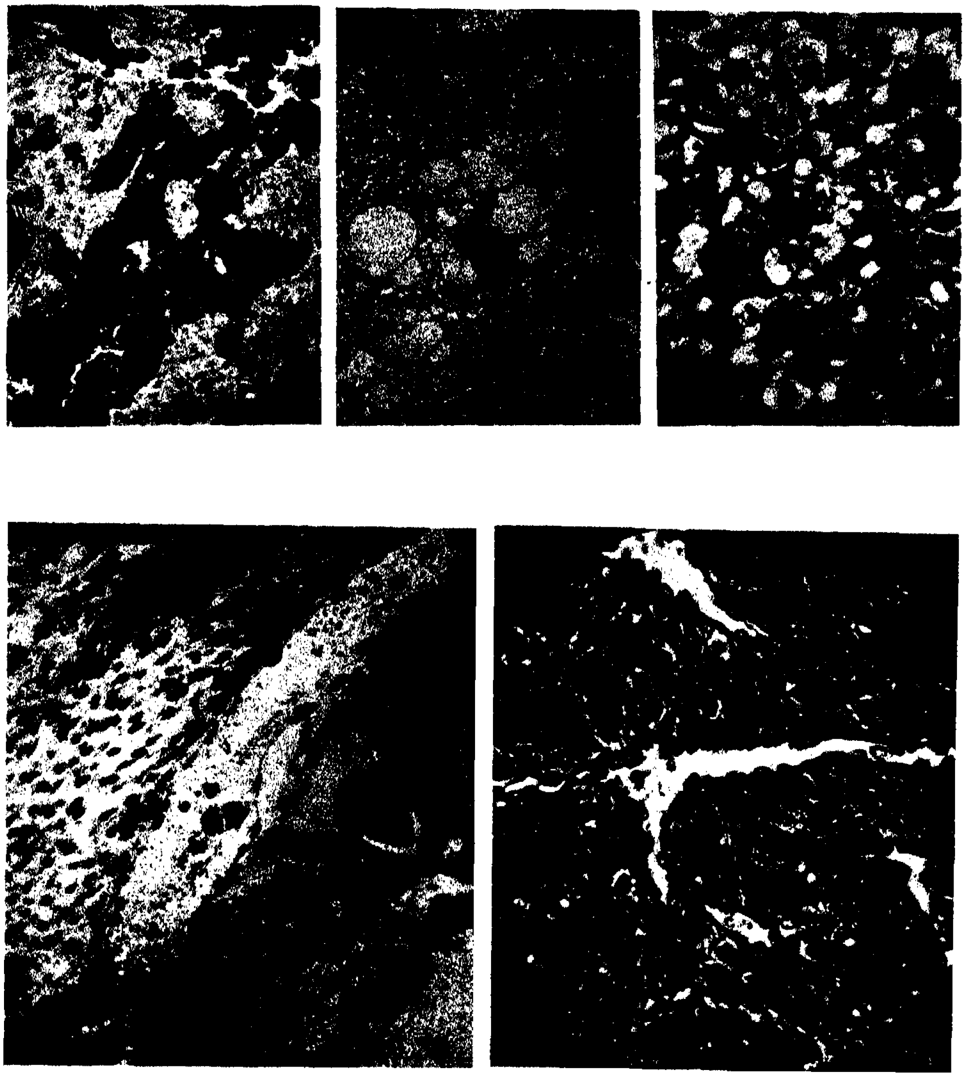
24

25

26







Since corpus luteum hormone inhibits the vaginal reaction to oestrin, and since we have no adequate test at the present time which would detect oestrin in the presence of corpus luteum hormone, we were interested to see whether this particular type of endometrial response to trauma could also be obtained if oestrin were given to an animal having a functional corpus luteum.

For this purpose we performed the following experiment. Four lactating rats were treated with 30 γ of oestrone daily, beginning on the 5th day of lactation, at which date one horn of the uterus was slit, as in the previous experiments. Biopsy on the 5th day thereafter showed that endometrial moles had formed under these circumstances and that they were larger than those obtained with oestrin in the ovariectomized females. There were no deciduomata. Vaginal oestrus was not produced by this treatment owing to the presence of the corpus luteum of lactation. We might mention that control experiments showed that at this stage of lactation slitting of the uterus invariably leads to the formation of deciduomata in untreated animals. That this reaction is not obtainable in rats only has been shown in three oestrous rabbits, all of which formed large moles 4 days after their uterus had been slit, fig. 10, Plate 2. It seems justifiable to conclude that the endometrial mole is a reaction to trauma dependent upon oestrin and particularly enhanced in the presence of an active corpus luteum. We think that by means of this reaction one may detect oestrin even though its vaginal effect be masked by the simultaneous presence of an active corpus luteum. It may be mentioned here that in other experiments we found that the daily administration of amounts of oestrone as large as 2000 to 3000 γ failed to elicit the vaginal response in the presence of the corpora lutea of lactation or pregnancy, while, as the present series shows, as small a dose as 30 γ produces unmistakable uterine reactions. We have purposely avoided in this discussion the statement that the corpus luteum hormone is responsible for the modification of the oestrin effects. Only experiments in which corpus luteum hormone and oestrin are given simultaneously to castrates would prove this. The present series of experiments may equally be explained by the assumption that oestrone is transformed by the corpus luteum into a derivative with modified physiological effects.

We hoped, using the formation of endometrial moles as a qualitative reaction of oestrin in the presence of corpus luteum hormone, to determine whether oestrin is produced during gestation in the rodent. In 12 rats one horn of the uterus was slit open between the 12th and 13th days of gestation, while the other horn with its gestation sacs was not touched. Biopsy specimens, taken 2 days later, showed that the uterine mucosa of these rats is much thicker on the traumatized side than usual, and histologically this thickening proved to be due to a hydrops of the stroma mucosae. Five days later the mucosa is enormously enlarged and transformed into a translucent gelatinous tumour with a more or less irregular surface. Histologically these tumours are typical endometrial moles. The formation of endometrial moles after trauma during the second half of gestation in the rat makes it very likely that oestrus-producing hormone is formed during pregnancy in the rodent.

The fact that the formation of endometrial moles is really dependent upon the quantity of oestrin present in the organism is shown still more clearly by the following experiment. In seven rats the uterus was slit on one side between the 12th and 13th days of gestation, and thenceforward the animals received 30 $\gamma$ of oestrone dissolved in corn oil daily, by subcutaneous injection. Biopsy on the 6th day after traumatization showed that the endometrial moles were larger in this group than in the previous one. These animals showed no vaginal oestrus. (The vaginal reaction was inhibited by the presence of the corpus luteum of gestation.)

We believe, accordingly, that the formation of endometrial moles during gestation proves the presence of oestrin in the circulation of the pregnant rodent. But where does this oestrin come from? Is the site of oestrin production during gestation in the ovary or in the placenta? The fact that the excretion of oestrin is hardly altered by ovariectomy makes it very likely that the placenta produces the large quantities of oestrin present in the pregnant woman. In order to establish the source of oestrin in the pregnant rat the following experiment was performed. In 10 rats at the 10th–12th day of gestation all the embryos were removed from the left horn, and the placentae left intact; the right horn of the uterus was slit open and embryos and placentae removed. At the same time complete ovariectomy was performed. The animals were killed 7 days later. The placentae in the left horn were still alive, and—judged by the fully developed and lactating condition of the mammary glands at the time of autopsy—apparently functional. The right horn, however, showed no sign of mole formation. This experiment indicates that in the pregnant rat, unlike the human, oestrin is formed by the ovary and not by the placenta.

We should like to call attention in this connexion to the experiments of Pencharz and Long (1931) and of Selye, Collip, and Thomson (1933), showing that in hypophysectomized pregnant animals gestation is not materially interfered with. This is only possible if the uterus does not involute after hypophysectomy as it does after oophorectomy; in fact, the uteri of the hypophysectomized pregnant rats displayed the second progestational reaction, which we have attributed to the combined action of oestrin and corpus luteum hormone, for reasons previously discussed. We concluded from these and other experiments (Selye, Collip, and Thomson, 1934) that a special gonadotropic hormone is produced by the rat placenta which acts on the ovary directly and not through the hypophysis. The present series of experiments would also seem to corroborate such an hypothesis, for the production of oestrin in the pregnant rat is not dependent on the presence of the hypophysis but on the simultaneous presence of the placenta and the ovary. It is likely, therefore, that the placenta produces a gonadotropic substance which stimulates the ovary to produce an excess of oestrin during gestation, and that this stimulus does not act through the pituitary but reaches the ovary directly. It is quite possible that this substance is simply an inactive precursor of oestrin which is transformed into the active substance only in the presence of the gonad.

These experiments give further support to the view expressed in the chapter on the physiology of the decidual mole, in which we explained the fact that one may not obtain deciduomata during the second half of gestation by the assumption that an excess of oestrin is produced at this time.

Before finishing this chapter we should like to call attention to the great similarity between the endometrial mole and the so-called stroma polyp of the endometrium as observed in the human subject. Both consist mainly of stroma mucosae and are often hydropic. Is it possible that these endometrial tumours, so frequently observed at the time of the menopause when oestrin is known to be produced in large quantities, could also be due to the simultaneous presence of excess oestrin and a local irritant in the uterus?

The question whether the endometrial mole bears any relation to the hydatidiform mole is still more problematic. Hydatidiform moles do, however, contain large quantities of oestrin, as shown by Philipp (1929), and it does not seem impossible that the placental stroma may react to oestrin in the presence of a local irritant in the same way as does the endometrium.

## III—The Metrial Gland

In the course of our experiments relating to the physiology of the placenta our attention has been drawn to an enlargement in the wall of the uterus just below the placenta. This region of the placental insertion contains many granulated epithelioid cells arranged around the blood vessels. Cells of a similar nature were probably first described by Minot in 1889 in the antimesometrial region of the pregnant rabbit uterus and later by Ulesko-Stroganowa (1896) and Maximow (1900). In 1911 Ancel and Bouin discovered large accumulations of similar cells in the subplacental region of the rabbit uterus, and these they named " glande endocrine myometriale." According to these authors, it appears in this species at about the 16th day of gestation, reaches a maximum of development on the 23rd day, and disappears shortly before the end of pregnancy. They believe these cells to be derived from the connective tissue elements situated between the muscle cells and suggest that they have an endocrine function. This latter statement, however, was based only on their morphological appearance. Two years later Frankel confirmed the observations of the French authors; but he did not always find the structure and therefore was not inclined to ascribe an important function to it. Discussing the origin of these cells, he states that their appearance makes it very probable that they are simply wandering cells which have emigrated from the foetal placenta. Weymeersch (1913) describes similar cells in the guinea-pig and attributes to them a phagocytic function. He calls them " nephrophagocytes." Similar formations have also been described by Keiffer (1925) under the name of " placenta myometrialis " in the guinea-pig, rabbit, and human; by Vercesi (1914) in the rabbit; by Weil (1919) in the rat; by Gérard (1925) in the mouse and rat; by Fornero (1924) in the human; by Muraoka (1920) and by Mossman (1926) in the rabbit, guinea-pig, rat, and human.

The epithelioid cells described by these various investigators are not all of the same type; Gérard, for instance, states definitely that the cells which he saw in the mouse and rat are not identical with those described by Bouin and Ancel in the rabbit. The fact that many of the workers quoted above ignored the publications of the others adds to the difficulty of correlating their findings.

The question of the origin of these cells is a controversial one. Are they of foetal origin, as Frankel, and more recently Robert Meyer, Sansom (1927), and Grosser (1927) thought, or are they derivatives of the intermuscular connective tissue of the mother, as Ancel and Bouin, Weil, Muraoka, Gérard, and others believe? Their function is also very problematical. Ancel and Bouin thought them to be endocrine cells, Weymeersch and others believed them to be phagocytes, while Gérard thinks they supply nutrition to the developing embryo.

Since this structure lies in both myometrium and mesometrium and since its endocrine function is not proved, we thought that it might lead to confusion to call it the endocrine gland of the myometrium. Furthermore, this name has been used for a certain type of cell found in the subplacental area of the rabbit uterus, although in our opinion the cells in this region are not essentially different from those in the antimesometrial wall of the uterus described by Minot (1890) under the name of "monster cells," nor from the large endothelial cells in the placenta materna. We shall therefore refer to this structure simply as "metrial gland."

During gestation the metrial gland begins to form at about the 8th–10th day in the rat. Since we described the histogenesis of this structure in connexion with deciduoma formation, we need not go into much detail here. The most important fact that we wish to establish in this connexion is this: the typical element of the active metrial gland of the rat, the granulated cell containing glycogen, eosinophilic, and sometimes also basophilic granules, may be formed from various sources; fig. 11, Plate 2, shows mitotic figures in the endothelium and the muscular coat of the insertion area in the rat. Typical granulated cells may be formed from both these sources. Fig. 12, Plate 2, shows the beginning of glycogen deposition in the cells of the muscular wall of the uterus. The cells in our figure are still spindle shaped; but all intermediate stages between this cell and the round or polygonal metrial gland cell may be found in the insertion area, so that the formation of granulated cells from the muscle cells of the uterus also appears very likely. Finally fibroblasts may also be transformed into granulated cells. This generalized metaplasia and active mitotic proliferation of various elements in the subplacental area into granulated metrial gland cells accounts for the enormous development of the metrial gland during the second half of gestation. It is possible that the decidua also takes part in the formation of the metrial gland, for we have frequently observed that strands of decidual cells invade the muscular wall of the uterus in the subplacental area around the central artery of the placenta, and frequently these strands extend far into the mesometrium, fig. 6, Plate 1. The decidua basalis itself consists of small non-vacuolated homogeneous cells, and it is only at those points at which the trophoblast has already invaded the structure that these decidua cells begin to store large quantities of glycogen, and so become similar to the metrial gland cells in and outside the muscular wall. The glycogen-containing decidua cells lying between the trabeculae of the small cellular trophoblast differ, however, from the metrial gland cells as they contain no eosinophilic or basophilic granules, figs. 13, 14, and 15, Plate 2.

In the rabbit the morphology of this region is only apparently different. The metrial gland cells are more homogeneous and rarely contain any glycogen, and the whole development of the metrial gland is far behind that of the rat, fig. 16, Plate 3. But this is compensated for by great development of the decidua, which consist only of glycogen-containing cells with no chromophil granules. The vascular endothelium is particularly well developed in the metrial gland of the rat, fig. 17, Plate 3, forming large cells which are often liberated into the blood-stream, but is usually not very well developed in the metrial gland of the rabbit, fig. 16, Plate 3. On the other hand, endothelial cells are very large in the vessels of the decidua basalis of the rabbit, figs. 18 and 19, Plate 3, while in the rat they show little development at this location, fig. 20, Plate 3. These endothelial cells protrude into the lumen of the arteries like polyps and are finally liberated into the blood-stream. Hence one may frequently find no endothelium on one side of a blood vessel and large endothelial cells on the other. The free endothelial cells are usually very similar to the typical granulated metrial gland cells, figs. 21, Plate 4, although they show no granules so long as they are in contact with the wall of the vessel from which they come. All these observations tend to show that the metrial gland cells are very similar to the cells of the decidua basalis, and it seems justifiable, therefore, to assume that the poor development of the metrial gland in the rabbit, as compared with that in the rat, is related to the better development of the decidua basalis in the former species. Since we believe, for reasons to be discussed later, that the function of the decidua basalis and of the metrial gland is the same, it seems obvious that if one of these two structures be very well developed there is no need for the development of the other.

We have already mentioned that in our opinion the " monster-cells " of Minot, fig. 22, Plate 4, in the mucosa and the muscular wall of the anti-mesometrial side of the uterus are also related to the metrial gland cells. How can one explain the presence of metrial gland cells on that wall of the uterus which lies opposite to the placental insertion ? Since such " monster cells " are never found in sections of those portions of the uterus between embryos, *i.e.*, at a place not in the vicinity of the ovum, it seems to us very likely that these cells are the result of the first nidation of the embryo. This in the rabbit takes place on the antimesometrial side. The decidual islands occasionally found in this region may also be explained in the same way.

We were able to obtain typical metrial glands in the lactating rat by mere mechanical irritation of the uterine mucosa. This fact definitely proves that it is not correct to consider the glands to be derivatives of the foetal ectoderm. They would appear to be simply the result of the mechanical or chemical stimulation of the endometrium, by the nidating ovum in pregnancy, and by the scratching of a needle in our experiments. It is essential for their formation, however, that a functional corpus luteum be present at the time when the uterine stimulus is applied, for it was impossible to produce metrial glands in experiments in which the endometrium was stimulated in the absence of an active corpus luteum.

We should also like to mention that the formation of the intervillous space is also, contrary to the common belief, due to factors in the maternal organism. In the experiments on the deciduoma mentioned above, we saw that at the time when the metrial gland is formed large venous sinuses form in the centre of the deciduoma. These sinuses unite into still larger venous spaces by dissolution of the decidual tissue which originally separated them, fig. 23, Plate 4. All this occurs in the absence of the foetal placenta. We feel that much of what has been said about the fermentative dissolution of maternal tissue by chorionic villi will have to be revised in view of these observations.

After the 8th day in artificially produced deciduomata and after delivery in normal gestation, the metrial gland begins to involute in the rat, figs. 24, 25, Plate 4, and fig. 26, Plate 5. We shall confine ourselves to the description of the changes taking place during the involution after delivery, since they are very similar to those seen in deciduomata undergoing retrogression.

A few days after delivery numerous vessels of the insertion area are thrombosed. The thrombi can frequently be identified as debris coming from the uterine wound in pregnancy, and from the degenerating decidual tissue in deciduoma involution. Many of the typical granulated cells of the metrial gland degenerate after delivery; others phagocytose hematogenous pigment derived from the blood of the thrombi, fig. 27, Plate 5. The iron content of this pigment has been demonstrated by means of the Prussian blue reaction. Still other granulated cells become loaded with lipoid material so that their appearance becomes very similar to that of the corpus luteum or adrenal cortex cells. The connective tissue around these lipoid-storing cells becomes more and more hyaline in appearance, figs. 28, 29, Plate 5. The development of these fat-storing cells and the surrounding hyaline tissue is more marked during lactation than it is if the young are weaned immediately following delivery. The development of the pigment storing phagocytes, however, is more or less independent of the nursing stimulus. These pigment cells are only very slowly removed from the metrial area. They form spots easily detectable by naked eye inspection and these mark the site of a previous gestation many months after delivery.

We believe that the metrial gland and the decidua basalis have more *than one function. The most important task is probably to furnish nutrition to the embryo.* The decidua basalis is directly digested by the trophoblast; the large endothelial cells, on the other hand, are first set free intact into the blood-stream, and are subsequently digested in the placental labyrinth. The metrial gland has to be considered as a holocrine, endocrine gland, using " endocrine " in the literal sense implying liberation of products into the blood-stream, without suggesting that any specific hormone is elaborated therein. Our experiments in which the embryos were removed from the uteri without interfering with the further development of the placenta have suggested a method by means of which this problem could be studied experimentally. In removing the embryo one removes the destination of the food products, particularly glycogen, prepared by the metrial gland. If it be true that the metrial gland cells provide nourishment for the embryo, then these cells should accumulate in the absence of the embryo. This is exactly what happens. Fig. 30, Plate 6, shows the large masses of glycogen-storing cells accumulating between the cells of the trophoblast in the placenta of a rat 5 days after the removal of the embryo. It seems that the production of these decidual cells is independent of the embryo, but in the absence of the latter they are not used and so accumulate in large quantities. It is interesting to note in this connexion that large and irregular venous sinuses form in the placental labyrinth after the removal of the embryo; yet the life span of the placenta is not materially influenced by this operation, in spite of the fact that the whole foetal circulation is abolished, figs. 31, 32, Plate 6. The vessels of the metrial gland contain numerous free metrial cells in these cases, apparently produced by the vascular endothelium and not used in the absence of the foetus, fig. 33, Plate 6. In the deciduoma this nutritive function of the metrial gland becomes still more evident. Here the blood vessels in the decidual tissue are usually simply crowded with granulated cells at the time when the insertion gland reaches its full development, fig. 34, Plate 6.

In addition to this nutritive function the metrial gland cells have the ability to phagocytose the debris of the placenta or of the degenerating deciduoma. These are brought in contact with them since at least part of this material is eliminated from the uterine cavity through the veins of this region. This phagocytic activity is responsible for the pigmented metrial cells, which are so frequently formed in the area of the placenta insertion after delivery.

We have been unable so far to determine any true hormonal function of these cells, although their special development during lactation is suggestive of some such activity.

In conclusion, it may be stated that our experiments show that the metrial glands do not form from the foetal ectoderm, as has been suggested, but from various maternal elements. They can only be formed in the presence of an active corpus luteum. The disintegration of decidual cells and the formation of large venous sinuses ready to receive the placental villi is largely independent of placental digestive ferments, since it also takes place in deciduomata in the absence of foetal cells. The " monster cells " of Minot, the " glande endocrine myometriale " of Bouin and Ancel, the decidua basalis, and the large endothelial cells of both the decidua and the metrial gland are all parts of the same organ system, the function of which is to nourish the ovum. It seems, therefore, that the blood of the mother is not the only source of nourishment for the foetus but that the metrial gland produces special substances, particularly glycogen, which are essential for embryonic growth. After delivery the metrial gland cells phagocytose much of the debris resulting from separation of the placenta. The significance of the unusual development of the metrial gland during lactation is not yet known. Since at this time these richly vascularized epithelioid cells are only in contact with the maternal blood, an internal secretory function may be suspected.

The authors are greatly indebted to Professor Girard of the University of Paris for supplying them with large quantities of oestrone used in these experiments.

## SUMMARY AND DISCUSSION

The cells of the deciduoma produced under experimental conditions in the absence of a nidating ovum are arranged in the same way as are those seen in the implantation site during normal pregnancy. The formation of the placenta on the mesometrial side is therefore determined by factors in the maternal organism, and is independent of the ovum and of the site of the trauma producing the reaction.

In artificially produced deciduomata a large blood sinus forms at a certain stage of development by liquefaction of the decidual cells between the blood vessels. This large blood sinus is analogous to the intervillous space of the placenta. Its formation in the absence of any foetal tissue shows the ferments of the chorionic villi not to be essential to the opening of maternal blood vessels.

In the sub-placental area the granulated cells considered by various authors as wandering cells derived from the foetal ectoderm are shown to be of maternal origin. This is proved by the observation that they may form in the vicinity of deciduomata in the absence of all foetal tissue.

*The formation of a deciduoma is inhibited during pregnancy by the* presence of the placenta, and during lactation by previously formed decidual tissue. If Caesarean section be performed during the early stages of gestation, deciduoma production becomes possible several days after the time at which it would have been impossible in the presence of placental tissue.

This factor inhibiting deciduoma production we believe to be oestrin. At the same period of pregnancy at which the uterus is converted to the second progestational stage it becomes impossible to obtain the decidual response. A similar morphological and physiological condition is observed in the endometrium of the lactating animal in the presence of decidual tissue.

Copulation leads to the establishment of a period of activity of the corpora lutea which is independent of the presence of the fertilized ovum. This is proved by the fact that the corpora lutea do not involute for several days after Caesarean section in the early stages of pregnancy.

We obtained deciduomata under various conditions leading to cachexia (adrenalectomy, starvation). These experiments make it likely that the observations of deciduoma production in animals during thyroid feeding and on vitamin deficient diets are to be explained as consequences of unspecific inhibition of follicle maturation.

It has been found that oestrin leads to the formation of large hydropic tumours in the traumatized endometrium of the castrate female rat. Still larger tumours of a similar type will form if the experiment be performed in the presence of an active corpus luteum. Structurally they resemble hydropic stroma polyps of the endometrium. Since their stroma is very similar to that of hydatidiform moles, they are referred to as the " endometrial mole."

During the second half of gestation, endometrial moles are invariably formed if one horn of the uterus in the rat be slit. We believe this to be additional evidence in support of our belief that oestrin is produced in the rodent during gestation.

No endometrial moles were obtained in the ovariectomized pregnant rat, although the placenta continued to grow in the absence of the gonad and the embryos. We conclude that in the rat, unlike the human, the oestrin produced during gestation does not come from the placenta but from the ovary. The partial involution of the uterus seen in the ovariectomized pregnant rat corroborates this view.

A histological investigation of the placental insertion area in the rat and rabbit leads to the conclusion that the " monster cells " of Minot, the " glande endocrine myometriale " of Bouin and Ancel, and the hypertrophied endothelial cells in the vessels of the decidua basalis, the mesometrial wall of the uterus and the adjacent mesometrium, are probably closely related to each other. They form an organ system which is concerned chiefly with the nutrition of the embryo. The name " metrial gland " is suggested for this organ system.

The endothelial cells of the metrial gland are liberated into the bloodstream, and in this way they furnish nourishment to the embryo. They are considered to be an holocrine-endocrine gland. The changes that take place in the placenta after the embryo, the consumer of these food substances, is removed furnish further evidence for the view that one of the most important functions of the metrial gland is to prepare the nourishment needed by the embryo.

## References

Allen, W. M. (1931). ' Anat. Rec.,' vol. **48**, p. 65.
—— (1932). ' Amer. J. Physiol.,' vol. **100**, p. 650.
Ancel, P., and Bouin, P. (1911). ' C.R. Ass. Anat. 3ième Réunion,' Paris.
Aschner, B. (1918). (Quoted by Biedl, Peters, and Hofstätter.)
Biedl, A., Peters, H., and Hofstätter, R. (1921). ' Z. Geburtsch. Gynäk.,' vol. **84**, p. 59.
Bishop, H. S., and Morgan, A. F. (1928). ' Proc. Soc. Exp. Biol. Med.,' vol. **25**, p. 438.
Brouha, A. (1932). ' C.R. Soc. biol. Paris,' vol. **110**, p. 1023.
Brouha, L. (1928). ' Proc. Soc. Exp. Biol. Med.,' vol. **25**, p. 488.
Cameron, G. R., and Amies, A. B. P. (1926). ' Austral. J. Exp. Biol. and Med. Sci.,' vol. **8**, p. 37.
Campbell, M., Wolfe, J. M., and Phelps, D. (1934). ' Proc. Soc. Exp. Biol. Med.,' vol. **32**, p. 205.
Corner, G. W., and Warren, S. L. (1919). ' Anat. Rec.,' vol. **16**, p. 168.
Courrier, R. (1930). ' C.R. Soc. biol. Paris,' vol. **104**, pp. 280, 282.
Courrier, R., and Kehl, R. (1930). ' C.R. Soc. biol. Paris,' vol. **104**, p. 1180.
Desclin, L., and Brouha, L. (1931). ' C.R. Soc. biol. Paris,' vol. **107**, p. 895.
Engle, E. T., and Shelesnyak, M. C. Unpublished data (referred to by Shelesnyak, 1933, *b*).
Evans, H. M. (1928). ' Amer. J. Physiol.,' vol. **85**, p. 149.
Evans, H. M., and Simpson, M. E. (1929). ' Proc. Soc., Exp. Biol. Med.,' vol. **26**, p. 597.
—— (1930). ' Anat. Rec.,' vol. **45**, p. 45 (supplement).
Fornero, A. (1924). ' Anat. Anz.,' vol. **58**, p. 553.
Frank, R. T. (1911). ' Surg. Gynec. Obstet.,' vol. **13**, p. 36.
Friedman, M. H. (1932). ' J. Pharmacol.,' vol. **45**, p. 7.
Gasbarrini, A. (1911). ' Int. Monatsschr. Anat. Physiol.,' vol. **28**, p. 250.
Gérard, P. (1925). ' C.R. Soc. biol. Paris,' vol. **93**, p. 457.
Goldstein, L. A., and Tatelbaum, A. J. (1930). ' Amer. J. Physiol.,' vol. **91**, p. 14.

Grosser, O. (1927). " Frühentwicklung, Eihautbildung und Placentation," J. F. Bergmann, Münich.

Hammond, J. (1917). ' Proc. Roy. Soc.,' B, vol. **89**, p. 534.

Hisaw, F. L., and Leonard, S. L. (1930). ' Amer. J. Physiol.,' vol. **92**, p. 574.

Innes, J. R. M., and Bellerby, C. W. (1929). ' J. Physiol.,' vol. **67**; Proc., p. xxxiv.

Keiffer, H. (1925). ' Bull. Anat. Roy. Méd. Belg.,' vol. **5**, p. 684.

Krainz, K. (1914). ' Arch. mikr. Anat.,' vol. **84**, p. 122.

Kutz, R. L., McKeown, T., and Selye, H. (1934). ' Proc. Soc. Exp. Biol. Med.,' vol. **32**, p. 331.

Leonard, S. L., Hisaw, F. L., and Fevold, H. L. (1932). ' Amer. J. Physiol.,' vol. **100**, p. 111.

Leonard, S. L., and Meyer. Unpublished data, referred to by Hisaw in " Sex and Internal Secretions."

Loeb, L. (1907). ' Zbl. allg. Path.,' vol. **18**, p. 563.

—— (1908). ' J. Amer. Med. Ass.,' vol. **50**, p. 1897.

Loeb, L., and Kountz, W. B. (1928). ' Amer. J. Physiol.,' vol. **84**, p. 283.

Long, J. A., and Evans, H. M. (1922). " Mem. Univ. of Calif."

Maximow, A. (1900). ' Arch. mikr. Anat.,' vol. **56**, p. 699.

Meyer, R. (1931). Henke-Lubarsch's " Handbuch der pathologischen Anatomie."

Minot, C. S. (1889). ' J. Morphol.,' vol. **2**.

—— (1890). ' Biol. Zbl.,' vol. **10**.

Mossman, H. W. (1926). ' Amer. J. Anat.,' vol. **37**, p. 433.

Muraoka, C. (1920). ' Frankf. Z. Path.,' vol. **22**, p. 208.

—— (1925). ' Kinki-Fujinkwa Gakkwow Zassi,' vol. **8**.

Nelson, W. O., and Pfiffner, J. J. (1930). ' Proc. Soc. Exp. Biol. Med.,' vol. **27**, p. 863.

Nielsen, F. (1921). ' C.R. Soc. biol. Paris,' vol. **85**, p. 368.

Parkes, A. S. (1929). ' Proc. Roy. Soc.,' B, vol. **104**, p. 183.

Pencharz, R. T., and Long, J. A. (1931). ' Science,' vol. **74**, p. 206.

Philipp, E. (1929). ' Zbl. Gynäk.,' vol. **53**, p. 2386.

Raynaud, R. (1934). " Sur l'Antagonisme fonctionnel entre les hormones ovariennes."

Reiss, M., and Pereny, S. (1928). ' Endocrinol.,' vol. **2**, p. 181.

Robson, J. M. (1935). ' Quart. J. Exper. Physiol.,' vol. **24**, p. 337.

Sansom, G. S. (1927). ' Proc. Roy. Soc.,' B, vol. **101**, p. 354.

Selye, H., Collip, J. B., and Thomson, D. L. (1933). ' Proc. Soc. Exp. Biol. Med.,' vol. **30**, p. 589.

—— (1934). ' Anat. Rec.,' vol. **58**, p. 139.

—— (1935). ' Endocrinol.,' vol. **19**, p. 151.

Selye, H., Harlow, C., and McKeown, T. (1935). ' Proc. Soc. Exp. Biol. Med. (*in the press*).

Selye, H., and McKeown, T. (1934). ' Proc. Soc. Exp. Biol. Med.,' vol. **31**, p. 683.

Shelesnyak, M. C. (1931). ' Amer. J. Physiol.,' vol. **98**, p. 387.

—— (1933, *a*). ' Endocrinol.,' vol. **17**, p. 578.

—— (1933, *b*). ' Amer. J. Physiol.,' vol. **104**, p. 693.

—— (1933, *c*). ' Anat. Rec.,' vol. **56**, p. 211.

Teel, H. M. (1926). ' Amer. J. Physiol.,' vol. **79**, p. 184.

Ulesko-Stroganowa, (1896). ' Mschr. Geburtsch.,' vol. **8** (quoted by Muraoka, 1920).

Van Horn, H. N. (1933). ' Endocrinol.,' vol. **17**, p. 152.

Vercesi, C. (1914). ‘Arch. ital, Biol.,’ vol. **62**, p. 421.
Weichert, C. K. (1928). ‘Proc. Soc. Exp. Biol. Med.,’ vol. **25**, p. 490.
—— (1930). ‘Physiol. Zool.,’ vol. **3**, p. 461.
Weichert, C. K., and Boyd, R. W. (1933). ‘Anat. Rec.,’ vol. **58**, p. 55.
Weil, P. (1919). ‘C.R. Soc. biol. Paris,’ vol. **82**, p. 1433.
Weymeersch, A. (1913–14). ‘Bull. Soc. belg. Gynec. Obstet.,’ vol. **24**, p. 28.

## DESCRIPTION OF PLATES

### PLATE 1

FIG. 1—Low magnification of rat uterus showing its general structure. Note the triangular area, limited on two sides by the mesometrial extension of the longitudinal and on the third side by the mesometrial surface of the circular muscle coat.

FIG. 2—High magnification of the epithelium lining the uterine cavity in a lactating rat. Note the lipoid granules in the basal surface of the cells (H.E. frozen section—stained with Sudan III).

FIG. 3—Low magnification of transverse section through the uterus of a deciduoma-bearing rat. Note the difference in structure between the mesometrial and the anti-mesometrial portions of the tumour, and the blood sinus formed under the surface epithelium.

FIG. 4—High magnification of mesometrial area of the deciduoma shown in fig. 3, consisting of small vacuolized cells.

FIG. 5—Anti-mesometrial area of deciduoma shown in fig. 3, consisting of large homogeneous cells.

FIG. 6—Transverse section through deciduoma showing the direct connexion between the decidua basalis and the insertion area. The central portion of the tumour has become necrotic.

FIG. 7—Transverse section through uterus of a lactating rat treated with oestrin. Compare with figs. 21 and 24, Plate 4, showing the uterus of lactating rats untreated; progestational proliferation stage 1. The endometrium has become hyperplastic, and shows the complicated lacing characteristic of the second progestational stage.

### PLATE 2

FIG. 8—Low magnification of large endometrial mole on the surface of the slit uterine horn. The wall of the uterus turned inside out as a result of the traction of its muscle cells. The great enlargement of the mucosa is due mainly to oedema.

FIG. 9—High magnification of a section from fig. 7, Plate 1, showing surface epithelium of the uterus mucosa and the oedematous stroma.

FIG. 10—Low magnification of endometrial mole formed in an oestric rabbit 3 days after the uterus had been slit.

FIG. 11—Blood vessel from the insertion area at the time of the formation of the metrial gland. Showing mitotic figures in the endothelium and the muscular wall.

FIG. 12—Beginning glycogen deposition in muscle cells of the insertion area (Best's Carmine method).

FIG. 13—Low magnification of the metrial gland. Practically every cell in this area has been transformed into the characteristic chromaphil granulated cell.

FIG. 14—High magnification of an area from fig. 13, showing typical chromaphil granulated cells.

FIG. 15—De-granulated cells between the strands of small cellular trophoblast in rat placenta. These cells are identical with those shown in fig. 14, differing only in that they contain no granules.

PLATE 3

FIG. 16—Homogeneous metrial gland cells (Bouin and Ancel's " glande endometriale ") from the sub-placental area of a rabbit. The endothelium is poorly developed.

FIG. 17—Cross-section of an artery in the metrial gland of the rat. The whole vessel is composed of granulated cells. Note the development of the endothelium.

FIG. 18—Low magnification of an area from the decidua basalis of the rabbit. The light decidual cells are loaded with glycogen. The endothelial cells are high cylindrical.

FIG. 19—High magnification of a section from fig. 18, showing large endothelial cells which protrude into the lumen.

FIG. 20—Cross-section of an artery from the decidua basalis of the rat. The endothelial cells are enlarged but not so much as in the rabbit, fig. 19.

PLATE 4

FIG. 21—A granulated cell floating free in a blood vessel of the metrial gland.

FIG. 22—" Monster cells " of Minot in the mucosa and muscular wall on the antimesometrial side of the uterus of a pregnant rabbit. Note the similarity between these giant cells and those from the subplacental area shown in fig. 16, Plate 3.

FIG. 23—Large blood sinus in the centre of a deciduoma corresponding to the intervillous space of the placenta. All the decidual vessels pour their blood into this central sinus which gradually enlarges by dissolution of the decidual tissue.

FIG. 24—Transverse section through insertion area of lactating rat 5 days after delivery showing hyalinization and partial involution of the gland.

FIG. 25—High magnification of insertion area from fig. 24. Note the light sheath of hyalinized stroma surrounding the blood vessels.

PLATE 5

FIG. 26—Macroscopic photograph of a uterine horn on the 4th day of lactation, showing the richly vascularized metrial glands.

FIG. 27—High magnification of an area from fig. 24, Plate 4, just below the endometrium, showing insertion cells loaded with granules of haematogenous pigment phagocytosed by insertion cells.

FIG. 28—High magnification of an area from fig. 24, Plate 4, showing lipoid-containing insertion cells surrounded by hyalinized connective tissue strands.

FIG. 29—High magnification of another area from fig. 24, Plate 4, farther away from the lumen of the uterus, showing insertion cells which have lost their granules, and have become loaded with lipoids. They resemble corpus luteum cells.

PLATE 6

FIG. 30—Large masses of glycogen-storing decidual cells between the cells of the small cellular trophoblast in the placenta of a rat 5 days after the removal of the embryo.

FIG. 31—Large irregular venous sinuses in the placental labyrinth of the rat 5 days after the removal of the embryo.

FIG. 32—Haematogenous pigment granules in the placenta of a rat 9 days after the removal of the embryo.

FIG. 33—Glycogen-storing insertion cells in a blood vessel of the decidua basalis in a rat 5 days after removal of the embryo. The giant cell in the wall of the blood vessel belongs to the large cellular trophoblast.

FIG. 34—Blood vessels in a deciduoma showing free insertion cells in the lumen.

---

575 · 31

# Transference of Induced Food-Habit from Parent to Offspring—II

By D. E. SLADDEN, D.I.C., Zoological Research Department, Royal College of Science

(*Communicated by E. W. MacBride, F.R.S.—Received May* 31, 1935)

## INTRODUCTION

The first part of this investigation was published in 1934 (Sladden, 1934). When that paper was read to the Society a discussion followed in which it was pointed out by critics that little or nothing was known of the habits of the stick-insect in its native country, India. In order to meet this criticism, the communicator of the paper (Professor E. W. MacBride, F.R.S.) instituted an extensive inquiry and obtained information from Northern, Central, and Southern India, from Ceylon, and also from Siam. The replies received give some idea of the local knowledge of the habits and systematics of this insect and supply information particularly relevant to the criticisms, although little work relating to this insect appears to have been done by Indian naturalists.

The ivy (*Hedera helix*), which was used as a food-plant in the experiments, was shown to be an inhabitant of Northern India; it clothes the middle slopes of the Himalayas; it is, in fact, a member of the Holarctic flora which descends to a level of about 2000 feet. On the southern slopes of this mountain range and at about this horizon the boundary between Holarctic and Oriental regions is drawn. A considerable number of plants (56) belonging to the ivy family (Araliaceae) are common in India, although none of them is closely allied to *Hedera* itself. About 20 plants of the Oleaceae, the family to which the privet (*Ligustrum*) belongs, are also found in India. A species of privet, it will be remembered, is the food-plant employed by breeders of the insect in Europe.

The only quarter from which satisfactory information was obtained was Madras. Dr. Gravely, the director of the Madras Museum, was able to give a clear account of the distribution of *Carausius*. Of this genus he distinguished two species, but he acknowledged that these were discriminated by colour, and since the previous work of MacBride and Jackson (1915) had shown colour to be extremely variable within the same species, the distinctions between the two species were not reliable. The types of the species *Carausius morosus* and *C. insolens* (probably one species) come from a range of hills south-west of Madras known as the Palni hills, at an altitude of about 4000 feet. At this altitude a more temperate climate prevails than in the rest of Southern India, which accounts for the fact that the stick-insects which are native to them will flourish in Europe.

On these hills there is a Jesuit training college, and Dr. Gravely discovered that some of the fathers were keen naturalists. One at least had been breeding *Carausius morosus* and sending the eggs to a fellow Jesuit in Europe. These insects were fed on *Acalypha indica*. It seems almost certain that it is through this source that the eggs have been reaching Europe.

In its native country *Carausius morosus* has never been recorded as feeding on ivy, but it has been seen attacking *Euonymus*. The ivy, however, grows well in this region, having apparently been introduced there as a garden plant. Besides the true ivy, two species of another plant (*Heptapleurum*) grow in these hills, and also *Schefflera* belonging to the same family but bearing no close resemblance to *Hedera*. So far as Fr. Anglade, the rector of the Jesuit college, could ascertain, none of the naturalists in Europe to whom the eggs were sent from the Jesuit college ever tried ivy as a food-plant.

## PARTICULARS OF EXPERIMENTS

The series of tests, both presentation and preference to which the insects in the following experiments were submitted, have already been fully described (Sladden, 1934). In that paper the experiments recorded extended over three generations. The present work continues the investigation for two further generations; five generations in all. The last two generations, generations 4 and 5, *cf.* Table I, have been submitted to a series of tests which are as follows:

A—Presentation tests on the offspring of ivy and privet-fed stock, 4th and 5th generations.

B—Preference tests on the offspring of ivy and privet-fed stock, 4th and 5th generations.

C—Presentation and preference tests on the offspring of ivy-fed stock (repeat of initial experiment).

D—Reversal test, presentation of privet to ivy-fed stock and to control stock.

It will be remembered that the essential differences between presentation and preference tests were briefly as follows. Presentation tests were tests in which the insect immediately on hatching was given ivy. This constituted the first presentation. If after a certain period the ivy was untouched, privet was offered. After one or more feeds of privet, ivy was again given, this being regarded as the second presentation, and so on until ivy was accepted, when only the ivy-feeding was continued.

In the preference tests, the insect on hatching was given both privet and ivy for three consecutive feeds. If it attacked both plants, it was regarded as " neutral," but if only one plant was accepted it was said to show a preference for that plant.

### A.1—PRESENTATION TESTS

Presentation tests 3 and 4 were carried out in 1933 and 1934 respectively. The insects submitted to the tests were the offspring of ivy-fed parents and are the third and fourth generations to be tested since the investigation began.

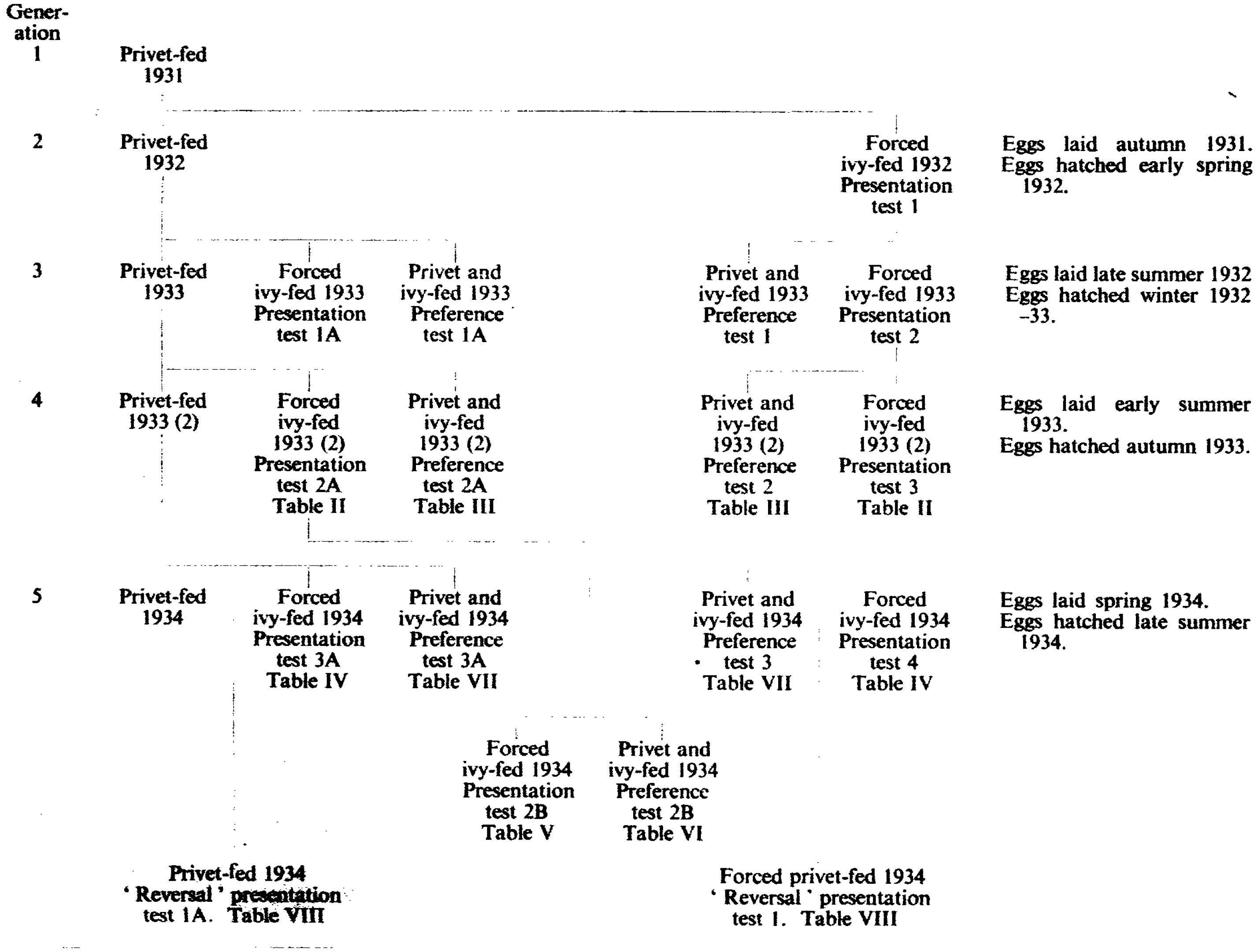
It will be remembered from the previous account that the parental insects were grouped together, all the individuals comprising a group being insects which accepted ivy at the same presentation.

In collecting eggs from a group it was found necessary to limit the number of eggs to an average of 200 per individual, *i.e.*, two individuals in the group, 400 eggs collected; three individuals, 600 eggs and so on.

This was also done in the control stock. This was permissible, as in the average insect the rate of egg-laying decreases considerably after the first 200–250 eggs are deposited.

Also towards the end of the egg-laying period a high percentage of eggs deposited are infertile. Table X gives the ratio of eggs selected,

TABLE I


Generation
1
Privet-fed 1931
2
Privet-fed 1932
Forced ivy-fed 1932 Presentation test 1
Eggs laid autumn 1931. Eggs hatched early spring 1932.
3
Privet-fed 1933
Forced ivy-fed 1933 Presentation test 1A
Privet and ivy-fed 1933 Preference test 1A
Privet and ivy-fed 1933 Preference test 1
Forced ivy-fed 1933 Presentation test 2
Eggs laid late summer 1932 Eggs hatched winter 1932–33.
4
Privet-fed 1933 (2)
Forced ivy-fed 1933 (2) Presentation test 2A Table II
Privet and ivy-fed 1933 (2) Preference test 2A Table III
Privet and ivy-fed 1933 (2) Preference test 2 Table III
Forced ivy-fed 1933 (2) Presentation test 3 Table II
Eggs laid early summer 1933. Eggs hatched autumn 1933.
5
Privet-fed 1934
Forced ivy-fed 1934 Presentation test 3A Table IV
Privet and ivy-fed 1934 Preference test 3A Table VII
Privet and ivy-fed 1934 Preference test 3 Table VII
Forced ivy-fed 1934 Presentation test 4 Table IV
Eggs laid spring 1934. Eggs hatched late summer 1934.
Forced ivy-fed 1934 Presentation test 2B Table V
Privet and ivy-fed 1934 Preference test 2B Table VI
Privet-fed 1934 ' Reversal ' presentation test 1A. Table VIII
Forced privet-fed 1934 ' Reversal ' presentation test 1. Table VIII

hatched, and used for certain groups of insects, and will be referred to again later. Thus in the majority of groups only the offspring produced in the earlier stages of the egg-laying period were used in the tests. The exceptions to this rule were groups of single individuals in which in some instances, owing to the small number of eggs deposited, every offspring hatched was tested (*e.g.*, Table II, group 2.3).

In all the presentation tests except test 2A, 150 eggs were selected at random from each parental group of eggs, and the first 100 offspring hatched were tested. Where this was not possible owing to the limited number of ova deposited, attention will be drawn to the fact. The

TABLE II

PRESENTATION TEST 3

| Ivy-fed parents 1933 Parental presentation group | No. tested | Presentations of ivy to progeny | | | | | Dead |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | |
| 1.1 | 100 | 99 | 1 | | | | |
| 1.2 | 100 | 93 | 7 | | | | |
| 2.1 | 100 | 91 | 8 | 1 | | | |
| 2.2 | 100 | 96 | 4 | | | | |
| 2·3 | 36 | 29 | 7 | | | | |
| 3.1 | 100 | 100 | | | | | |
| 3.2 | 100 | 90 | 10 | | | | |
| 3.3 | 100 | 96 | 4 | | | | |
| 4.1 | 100 | 96 | 4 | | | | |
| 4.2 | 100 | 95 | 5 | | | | |
| 5.1 | 100 | 93 | 7 | | | | |
| 5.2 | 100 | 99 | 1 | | | | |
| 5.3 | 100 | 90 | 10 | | | | |
| 6.1 | 100 | 93 | 7 | | | | |
| 6.2 | 100 | 95 | 5 | | | | |
| 6.3 | 100 | 97 | 3 | | | | |
| 6.4 | 100 | 91 | 9 | | | | |
| 7.1 | 100 | 91 | 9 | | | | |
| 7.2 | 100 | 94 | 6 | | | | |
| 7.3 | 100 | 93 | 7 | | | | |
| 10.1 | 100 | 89 | 9 | 2 | | | |
| 10.2 | 100 | 99 | 1 | | | | |
| 10.3 | 100 | $85^1$ | 14 | | | | 1 |
| Total............ | 2236 | 2095 | 138 | 3 | | | 1 |
| Percentage ...... | 100 | 93·7 | 6·2 | 0·1 | | | 0·04 |

Raised figure indicates number dead after refusing previous presentation.

TABLE II—(continued)

PRESENTATION TEST 2A

| Privet-fed parents 1933 | No. rested | Presentations of ivy to progeny | | | | | Dead |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | |
| | 421 | $227^1$ | $171^1$ | 20 | | | 3 |
| Total (%) ...... | 100 | 54·2 | 40·9 | 4·8 | | 0·2 | 0.7 |

Raised figures indicate number dead after refusing previous presentation.

selection of eggs required was not made until the total number of eggs per group had been deposited.

*Presentation Test* 3—*Table II*—Twenty-three parental groups, that is groups selected from the previous generation of ivy-fed stock, were used. After testing, a number of individuals from each group was reared to maturity in order to provide eggs for the next generation. The number of individuals in the parental groups in this case ranged from one to five. A total of 2236 offspring were used. (Group 2.3 contained only one insect and only 102 ova were deposited. Of these 36 hatched.) The comparatively high percentage of insects, 93·7%, readily accepted ivy at the first presentation, 6·2% took ivy at the second presentation, and 0·1% at the third. Only one insect (0·04%) of the 2236 died without accepting ivy.

*Presentation Test* 4—*Table IV*—The insects in this test represent the fifth generation. They are the offspring of certain groups of insects selected from the fourth generation submitted to presentation test 3. Only four groups were selected. Each group contained three individuals reared to maturity with the exception of group 6.4.2 in which there were only two. As before and in all subsequent tests, 200 eggs per individual were collected. Four hundred offspring were tested and of these 1 (0·25%) died untested. Of the remaining 399, 90·3% accepted ivy at the first presentation, 9·5% at the second, and 0·3%, or only one insect, at the third.

### A.2—CONTROL PRESENTATION TESTS

The results enumerated above should be compared with the corresponding control tests on the offspring of privet-fed stock, fourth and fifth generations, *cf.* Table I.

*Presentation Test* 2A—*Table II*—There were eight parental individuals reared to maturity from the privet stock in 1933, and 1600 ova were

TABLE III

PREFERENCE TEST 2

| Ivy-fed parents 1933 Parental presentation group | No. tested | Privet | Neutral | Ivy | Dead |
|---|---|---|---|---|---|
| 1.1 | 50 | 10 | 16 | 24 | 0 |
| 1.2 | 50 | 4 | 16 | 30 | 0 |
| 2.1 | 50 | 11 | 26 | 13 | 0 |
| 2.2 | 50 | 7 | 19 | 24 | 0 |
| 3.1 | 50 | 12 | 25 | 13 | 0 |
| 3.2 | 50 | 8 | 18 | 24 | 0 |
| 3.3 | 50 | 5 | 19 | 26 | 0 |
| 4.1 | 50 | 11 | 22 | 17 | 0 |
| 4.2 | 50 | 4 | 18 | 28 | 0 |
| 5.1 | 50 | 10 | 17 | 23 | 0 |
| 5.2 | 50 | 9 | 22 | 19 | 0 |
| 5.3 | 50 | 4 | 13 | 33 | 0 |
| 6.1 | 50 | 10 | 25 | 15 | 0 |
| 6.2 | 50 | 9 | 18 | 23 | 0 |
| 6.3 | 50 | 9 | 16 | 25 | 0 |
| 6.4 | 50 | 11 | 24 | 15 | 0 |
| 7.1 | 50 | 5 | 20 | 25 | 0 |
| 7.2 | 50 | 7 | 23 | 20 | 0 |
| 7.3 | 50 | 9 | 22 | 19 | 0 |
| 10.1 | 50 | 9 | 22 | 19 | 0 |
| 10.2 | 50 | 6 | 20 | 23 | 1 |
| 10.3 | 50 | 12 | 18 | 19 | 1 |
| Total.............. | 1100 | 182 | 439 | 477 | 2 |
| Percentage ........ | 100 | 16·5 | 39·9 | 43·4 | 0·2 |

PREFERENCE TEST 2A

| Privet-fed parents 1933 | No. tested | Privet | Neutral | Ivy | Dead |
|---|---|---|---|---|---|
| Total .............. | 250 | 83 | 110 | 57 | 0 |
| Percentage ........ | 100 | 33·2 | 44·0 | 22·8 | 0·0 |

collected. Five hundred eggs were selected at random and from these 421 offspring were tested. Of these only 54·2% accepted ivy at the first presentation, 40·9% at the second, and 4·8% at the third. Three insects (0·7%) died, one before the first presentation of ivy, one after refusing ivy at the first presentation, and the third after refusing ivy for four presentations.

*Presentation Test* 3A—*Table IV*—This was the control to presentation test 4, and was carried out in 1934 with the offspring of privet-fed stock, fifth generation. There were six individuals reared to maturity in the privet stock in 1934, and 1200 eggs were collected. One hundred offspring were tested and none of these died. A slightly higher percentage, 61·0%, than in the control test of the previous generation accepted ivy at the first presentation and a correspondingly lower number, 39·0%, took ivy at the second presentation.

TABLE IV

PRESENTATION TEST 4

| Ivy-fed parents 1934 Parental presentation group | No. tested | Presentations of ivy to progeny | | | | | Dead |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | |
| 1.1.1 | 100 | 96 | 3 | 1 | | | 0 |
| 2.3.1 | 100 | $82^{1}$ | 17 | | | | 1 |
| 6.4.2 | 100 | 96 | 4 | | | | 0 |
| 10.3.2 | 100 | 86 | 14 | | | | 0 |
| Total.............. | 400 | 361 | 38 | 1 | | | 1 |
| Percentage ........ | 100 | 90·3 | 9·5 | 0·3 | | | 0·25 |

PRESENTATION TEST 3A

| Privet-fed parents 1934 | No. tested | Presentations of ivy to progeny | | | | | Dead |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | |
| Total (%) ........ | 100 | 61 | 39 | | | | 0 |

Raised figure indicates number dead after refusing previous presentation.

## B.1—PREFERENCE TESTS

The insects submitted to preference tests 2 and 3 were the offspring of ivy-fed parents of the 4th and 5th generations. These tests were made in 1933 and 1934 at the same time and with offspring of the same parental groups as presentation tests 3 and 4 described above, *cf.* Table I. In all the preference tests, except test 2A, 100 eggs were selected from each parental group and the first 50 offspring hatched were tested for preference for food-plant, privet or ivy.

*Preference Test* 2—*Table III*—There were only 22 parental groups in this test instead of 23 as in presentation test 3. All the young hatched from group 2.3 were used in the presentation test of that generation. One thousand one hundred offspring were submitted to the test and of these 2 (0·2%) died untested, without having accepted privet or ivy. Of the remainder, the majority, 43·4%, preferred ivy, 39·9% were neutral, accepting either plant impartially, and only 16·5% preferred privet.

*Preference Test* 3—*Table VII*—From the insects of the fourth generation four parental groups were selected from those submitted to presentation test 3 to provide offspring for this test. It should be noted here that none of the insects submitted to preference tests was reared to maturity. Group 6.4.2 contained two individuals reared to maturity, the remaining groups three each. A total of 200 young were tested and none of these died. The results are comparable with those of generation 4, preference test 2. 47·0% of the offspring preferred ivy, 39·0% ate either plant, and 14·0% chose privet. Compared with the result of the parental generation, preference test 2, a slightly greater percentage preferred ivy.

### B.2—Control Preference Tests

Preference tests on the offspring of privet-fed stock, fourth and fifth generations were carried out in 1933 and 1934 as controls to preference tests 2 and 3, *cf.* Table I.

*Preference Test* 2A—*Table III*—One thousand, six hundred eggs were collected from the privet-fed stock of eight individuals reared to maturity. Five hundred ova were selected and the first 250 offspring hatched were tested. Among these there were no deaths. The results of the control test should be compared with its corresponding preference test, Table III, on ivy-fed stock, when it will be seen that while the majority of the young, 44·0%, were neutral, 33·2% preferred privet, and only 22·8% chose ivy.

*Preference Test* 3A—*Table VII*—From the 1200 eggs collected from the six individuals reared to maturity in the privet stock in 1934, 50 offspring were tested. None of the young died. As in the previous generation of the control, the greater number of offspring, 46·0%, were neutral, 28·0% preferred ivy, and 26·0% chose privet. In this test, while the greater percentage was neutral as before, there is no very marked difference between the number taking ivy and those taking privet. A slight preference for ivy, however, is indicated.

### C.1—Repeat Presentation Test

It was decided to repeat presentation test 2 (*see* Sladden, 1934, Table IV), using the offspring of another generation to see if the results were in any way comparable.

*Presentation Test* 2B—*Table V*—The offspring of ivy-fed stock, derived from certain insects of the privet-fed stock submitted to presentation test 2A, 1933, Table II were used. There were three parental groups, group 1 contained four individuals reared to maturity, and groups 2 and 3 three each. Eight hundred eggs were collected from group 1, and 600 from each of groups 2 and 3. A total of 300 young were tested, among which there were no deaths. The results of this test should be compared with those of presentation test 2A, to which the parental generation was submitted, when it will be seen that in the filial generation 85·7% accepted ivy at the first presentation, 14·0% at the second, and only 0·3% at the third. The results of the test on the parental generation were 53·9% at the first presentation, 40·8% at the second, and 4·7% at the third.

TABLE V—PRESENTATION TEST 2B

| Ivy-fed parents 1934 Parental presentation group | No. tested | Presentations of ivy to progeny | | | | | Dead |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | |
| 1 | 100 | 80 | 20 | | | | 0 |
| 2 | 100 | 86 | 13 | 1 | | | 0 |
| 3 | 100 | 91 | 9 | | | | 0 |
| Total.............. | 300 | 257 | 42 | 1 | | | 0 |
| Percentage ........ | 100 | 85·7 | 14·0 | 0·3 | | | 0·0 |

## C.2—REPEAT PREFERENCE TEST

A preference test was also carried out at the same time and with offspring of the same stock as presentation test 2B, just described. The parental groups and number of eggs collected were the same for both tests.

*Preference Test* 2B—*Table VI*—A total of 150 insects were tested and none of these died. The results of this test should be compared with those of preference test 2A, made with the parental generation, the results of which were 33·2% privet, 44·0% neutral, and 22·8% ivy. The filial generation when tested gave the following results, 9·3% privet, 29·3% neutral, and 61·3% ivy.

## D—REVERSAL TEST

After three generations of insects reared on ivy, a reversal test was devised. In this test the offspring of ivy-fed parents were presented with privet. The method of testing was the same as that used in all presentation tests except for the reversed use of the food-plants.

TABLE VI—PREFERENCE TEST 2B

| Ivy-fed parents 1934 Parental presentation group | No. tested | Privet | Neutral | Ivy | Dead |
|---|---|---|---|---|---|
| 1 | 50 | 5 | 13 | 32 | 0 |
| 2 | 50 | 3 | 17 | 30 | 0 |
| 3 | 50 | 6 | 14 | 30 | 0 |
| Total .............. | 150 | 14 | 44 | 92 | 0 |
| Percentage ........ | 100 | 9·3 | 29·3 | 61·3 | 0·0 |

*Reversal Presentation Test* 1—*Table VIII*—For this test four parental groups were selected from groups of insects submitted to presentation test 3. Each parental group consisted of three individuals reared to maturity. A total of 400 offspring were tested and among these there were no deaths. The insects readily accepted privet, 94·5% at the first presentation and 5·5% at the second.

TABLE VII

PREFERENCE TEST 3

| Ivy-fed parents 1934 Parental presentation group | No. tested | Privet | Neutral | Ivy | Dead |
|---|---|---|---|---|---|
| 1.1.1 | 50 | 8 | 24 | 18 | 0 |
| 2.3.1 | 50 | 5 | 16 | 29 | 0 |
| 6.4.2 | 50 | 11 | 24 | 15 | 0 |
| 10.3.2 | 50 | 4 | 14 | 32 | 0 |
| Total .............. | 200 | 28 | 78 | 94 | 0 |
| Percentage ........ | 100 | 14·0 | 39·0 | 47·0 | 0·0 |

PREFERENCE TEST 3A

| Privet-fed parents 1934 | No. tested | Privet | Neutral | Ivy | Dead |
|---|---|---|---|---|---|
| Total .............. | 50 | 13 | 23 | 14 | 0 |
| Percentage ........ | 100 | 26·0 | 46·0 | 28·0 | 0·0 |

*Control Reversal Presentation Test* 1A—*Table VIII*—As a control to the above test, the offspring of privet-fed parents were presented with

TABLE VIII

REVERSAL PRESENTATION TEST 1

| Ivy-fed parents 1934 Parental presentation group | No. tested | 1 | 2 | 3 | 4 | 5 | Dead |
|---|---|---|---|---|---|---|---|
| 1.1.1 | 100 | 97 | 3 | | | | 0 |
| 2.3.2 | 100 | 94 | 6 | | | | 0 |
| 6.4.1 | 100 | 92 | 8 | | | | 0 |
| 10.3.1 | 100 | 95 | 5 | | | | 0 |
| Total.............. | 400 | 378 | 22 | | | | 0 |
| Percentage ........ | 100 | 94·5 | 5·5 | | | | 0·0 |

CONTROL REVERSAL PRESENTATION TEST 1A

| Privet-fed parents 1934 | No. tested | Presentations of privet to progeny 1 | 2 | 3 | 4 | 5 | Dead |
|---|---|---|---|---|---|---|---|
| Total (%) ........ | 100 | 92 | 8 | | | | 0 |

privet. Of the 100 young submitted to this test, 92·0% accepted privet at the first presentation and 8·0% at the second; there were no deaths.

## DISCUSSION

The conclusion reached in the first paper (Sladden, 1934) was that the offspring of stick-insects reared from hatching on ivy, showed a greater ability to feed on ivy, and even a greater preference for it, than did their parents or control individuals of the same generation derived from privet-fed stock. This conclusion has been confirmed and strengthened by the results recorded in the present paper.

Examination of Table IX shows that this conclusion is true generally for the experimental generations. The fourth filial generation of the ivy-fed strain do show, it is true, a very slight decrease over the previous generation, but this cannot be regarded as significant as very many fewer individuals were tested owing to other experiments being in progress which necessitated a reduction in the large numbers previously dealt with. The preference test of that generation continues to show the increased preference for ivy.

A more significant variation is, however, to be found in the consideration of the privet-fed generation submitted to both presentation and preference tests. It will be noted that in both these series of tests there is a distinct gradual increase in the ability to eat and preference for ivy.

This increase is not so pronounced as in the ivy-fed forms. If it were, no conclusion such as I have drawn would be permissible. But it is, nevertheless, a fact that in four generations in the conditions under which they have been bred and reared these privet-fed individuals have attained an ability to eat and a preference for ivy very nearly equal to that possessed by the first generation of offspring from forced ivy-fed insects.

While this in no way weakens the case for a transmission of an ivy preference, some possible explanation may be suggested. The one feature of the conditions under which all the insects were bred which varied from generation to generation was the period (season) of the year at which they hatched and were subsequently reared. Reference to Table I will show that the length of the life-cycle, from egg to egg, was about 9–10 months. This naturally resulted in a setting back by 2·3 months of the hatching and rearing seasons. Now, although the foodstuffs were in a slightly different condition from generation to generation, this applies equally well to both the forced ivy-fed and privet-fed series.

TABLE IX

SUMMARY OF PRESENTATION TESTS

| Filial generation | Privet-fed parents | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1 | 8·0 | 32·0 | 21·6 | 12·0 | 11·2 | 11·2 | 2·4 | 0·8 | 0·0 | 0·8 |
| 2 | 30·7 | 46·0 | 14·7 | 2·0 | 3·3 | 0·7 | 0·0 | 0·0 | 0·7 | |
| 3 | 54·2 | 40·9 | 4·8 | 0·0 | 0·2 | | | | | |
| 4 | 61·0 | 39·0 | | | | | | | | |

| Filial generation | Ivy-fed parents | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1 | | | | | | | | | | |
| 2 | 78·25 | 18·99 | 1·5 | 0·12 | 0·12 | | | | | |
| 3 | 93·7 | 6·2 | 0·1 | | | | | | | |
| 4 | 90·3 | 9·5 | 0·3 | | | | | | | |

SUMMARY OF PREFERENCE TESTS

| Filial generation | Privet-fed parents | | | Ivy-fed parents | | |
|---|---|---|---|---|---|---|
| | Privet | Neutral | Ivy | Privet | Neutral | Ivy |
| 2 | 44 | 35 | 21 | 28·21 | 37·0 | 35·61 |
| 3 | 33·2 | 44·0 | 22·8 | 16·5 | 39·9 | 43·4 |
| 4 | 26·0 | 46·0 | 28·0 | 14·0 | 39·0 | 47·0 |

Any differences derived from these seasonal variations, therefore, would probably be found in the food preferences of the insect. No direct evidence is available that the acceleration of a yearly cycle to a 10-months cycle, or similar change in any other type of life-history, may produce changes in the food-habit of an insect or other animal, although the advantages of such a change in habit would be obvious. Possibly, too, an insect presented with a new food-plant would more readily adapt itself to it if the leaves were young and tender rather than old. This may account for the high percentage of offspring of privet-fed parents accepting ivy at the first presentation shown in experiment 2A. However, this explanation is merely tentative, and it is proposed to submit this point to further investigation

A further result may be noted in this connexion. The presentation and preference tests 2B, which are in nature a repetition of presentation test 2 and preference test 1, again show a marked increase in ability to eat, and preference for, ivy, not only over their parents (presentation and preference tests 2A) but also over the privet-fed offspring belonging to the same generation (presentation and preference tests 3A). This can only be explained on the grounds that the forced ivy feeding of their parents has been effective in the offspring whatever may be the nature of the slow and gradual increase in the ability to eat, and preference for, ivy apparent in the privet-fed stock.

The next point for comment is the very distinct slowing down in the rate of increase in the ability to eat ivy as shown clearly by the presentation tests. The enormous jump from 8·0% at the first presentation in the first filial generation to 78·25% in the second filial generation is very remarkable. Yet this is only increased to 93·7% in the third filial generation and decreases insignificantly in the fourth. This suggests that perhaps some limit is reached at about this figure, or more probably that the residual individuals, some 6–8%, represent an error inherent in the method of the experiment. This latter view received support from the reversal presentation test 1A, when 8% of the offspring of privet-fed parents refused privet at the first presentation. It seems possible that the method of presentation may have some inherent error, which should be corrected mathematically to attain a true representation of feeding habits. Such an error would appear most prominently when the first presentation group approximated to 100%. This does not seem to be a very serious objection to such conclusions as I have drawn from the figures.

Another point arising from the reversal presentation test is that the ivy-fed insects, while exhibiting an increased preference for ivy to privet, still retain the same ability to eat privet.

In conclusion, attention is drawn to Table X in which is set out a number of records of the eggs selected, hatched, and used from various groups of parents. These are in no way selected for consistency but rather that the data for these groups have been kept complete and are therefore capable

TABLE X—PERCENTAGES OF HATCHED AND USED EGGS

| Parental group | Eggs selected | Eggs hatched | Eggs used | % Selected/ hatched | % Selected/ used |
|---|---|---|---|---|---|
| 1.1.1 | 400 | 358 | 250 | 89·5 | 69·8 |
| 1.2.2 | 400 | 377 | 250 | 94·3 | 66·3 |
| 2.1.1 | 400 | 361 | 250 | 90·3 | 69·2 |
| 2.3.2 | 150 | 124 | 100 | 82·7 | 80·6 |
| 4.1.1 | 400 | 368 | 250 | 92·0 | 67·9 |
| 6.4.1 | 150 | 144 | 100 | 96·0 | 68·8 |
| 6.4.2 | 250 | 241 | 150 | 96·4 | 62·2 |
| 7.1.1 | 400 | 367 | 250 | 92·0 | 68·1 |
| 10.1.1 | 300 | 271 | 250 | 90·3 | 92·2 |
| 10.3.1 | 150 | 132 | 100 | 80·0 | 75·8 |
| 10.3.2 | 250 | 180 | 150 | 72·0 | 83·3 |
| Controls .......... | 800 | 737 | 500 | 92·1 | 67·8 |
| Totals ...... ...... | 4050 | 3660 | 2600 | 90·4 | 71·0 |

of exact treatment. It will be seen that the high average percentage hatch of 90·4% indicates favourable conditions of rearing and treatment, and that the average percentage of those used, 71·0%, does little more than ensure sufficient eggs with which to carry out the experiments. In other words, the range of percentage hatches is 72·0% to 96·4% and consequently not more than the lower of these two figures could be *relied on* to provide the adequate number of emerging insects.

I wish to express my sincere gratitude to Professor E. W. MacBride, F.R.S., who has supervised the work and given me every encouragement, and to Mr. H. R. Hewer, M.Sc., for advice and assistance, particularly in the preparation of the manuscript. I am deeply indebted to Professor B. K. Das, D.Sc., of the Osmania University, Hyderabad, Dr. W. Fernando, Lecturer in Zoology in the University of Colombo, and Dr. Gravely, superintendent of the Madras Museum, for the trouble they took in collecting information for me.

## SUMMARY

Experiments with the stick-insects (*Carausius morosus*), in which the insects for several generations were forced to accept a new food-plant (ivy) and then reared to maturity on it, have shown that the offspring of each subsequent generation accepted the ivy more readily than did their parents, and even showed an increased preference for it.

The offspring of each generation were submitted to presentation and preference tests already fully described.

The increasing ability of offspring of ivy-fed parents to eat ivy indicated in experiments previously recorded is still maintained, though the rate of increase is slowing down, as is clearly shown by the presentation tests. Reasons for this are discussed.

The preference tests continue to show that the insects have an increased preference for ivy.

## REFERENCES

MacBride, E. W., and Jackson, A. (1915). 'Proc. Roy. Soc.,' B, vol. **89**, p. 109.
Sladden, D. E. (1934). 'Proc. Roy. Soc.,' B, vol. **114**, p. 441.

577 . 158 . 52

# Studies in Peroxidase Action
## I—The Oxidation of Aniline

By PHILIP JOCELYN GEORGE MANN, Beit Memorial Research Fellow, and BERNARD CHARLES SAUNDERS, Charles Kingsley Lecturer in Natural Sciences, Magdalene College, Cambridge

(From the Biochemical Laboratory, Cambridge, and the University Chemical Laboratory, Cambridge)

(*Communicated by Sir F. G. Hopkins, O.M., P.R.S.—Received July* 12, 1935)

The production of colours by the action of the enzyme peroxidase on various amino and phenolic compounds has been recorded from time to time, but such reactions have, in general, been regarded merely as tests for the presence of peroxidase, and the nature of the coloured products has been determined only in a few instances. The fairly wide distribution of the enzyme, more particularly in plants, suggests that it has considerable biological importance, but the role that it plays in metabolic processes has not yet been elucidated. From the work of Elliott (1932, *a*, *b*), it would appear that the enzyme is not concerned with general oxidative katabolism since it does not attack the majority of amino-acids, fatty acids, and carbohydrates. On the other hand, where the products of oxidation have been investigated, they have proved to be, in general, of higher molecular weight than the substrate. Thus, for example, guaiacol is oxidized to tetraguaiacol (Bertrand, 1903), pyrogallol to purpurogallin (Willstätter and Heiss, 1923), and *o*-phenylene diamine to 2:3-diaminodiphenazine (Chodat, 1925).

It may well be an inherent property of the molecule which is causing condensation in the above cases, and it does not therefore necessarily follow that the enzyme is facilitating coupling of the substrate molecules as well as causing oxidation.

It is proposed to investigate the action of peroxidase on a range of relatively simple compounds, and then from a consideration of the types of change brought about to attempt the synthesis of compounds of biological importance.

The substrate chosen for the initial investigation is aniline. The first reference to the enzymatic oxidation of aniline appears to be that by Bouquelot (1896). He dissolved aniline in dilute acetic acid, mixed it with a small quantity of an infusion of " Russula delica," and on passing a current of air through the liquid obtained a magenta-like substance. It is obvious, however, that the enzyme used in this instance was tyrosinase.

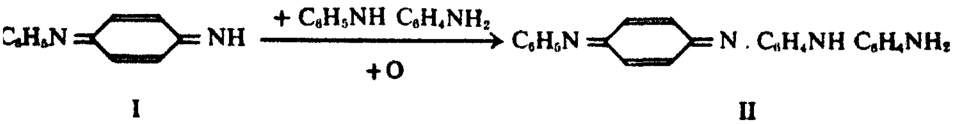
The enzyme preparations which we employed were derived either from horseradish or from turnips by the methods of Willstätter and Stoll (1918) and Elliot (1932, *b*). Identical oxidation products were obtained from aniline with each type of preparation. In control experiments no action on aniline was shown by the active enzyme preparation in the absence of hydrogen peroxide or by heat inactivated enzyme in the presence of hydrogen peroxide. It therefore appears justifiable to conclude that the product obtained was the result of peroxidase action.

Our experiments were carried out at room temperature, using a 2% solution of aniline in dilute acetic acid at $p_H$ 4·5. A few milligrams of the peroxidase preparation were added and dilute hydrogen peroxide was also added at intervals over a period of about 12 hours in such amounts that its concentration was maintained between 10 and 20 mg %. This intermittent addition of hydrogen peroxide is necessary, since, as was first shown by Bach (1904), excess of it inhibits peroxidase action. Further small amounts of the enzyme preparation were also added from time to time. The first noticeable effect was the formation of a blue-violet colour which rapidly changed to brown. This blue-violet colour was produced with each subsequent addition of hydrogen peroxide, and reasons are given later for supposing it to be due to the transient existence of quinone-anil-(4 (4-amino-anilino) anil). A brown solid gradually separated which was filtered off at the conclusion of the reaction. The filtrate was made alkaline, whereby a further small quantity of the bases of the same substances was obtained. The product proved to be a complex mixture of related substances which, by the method described later, was resolved into 2: 5-dianilino-*p*-benzoquinone imide-anil, induline, pseudo-mauveine, and ungreenable aniline black.

The separation of these substances proved a matter of some difficulty owing to their chemical similarity. The series of solvents ultimately used provided an efficient separation, but it is not improbable that traces of other substances may have been produced during the oxidation.

Neither benzoquinone nor azobenzene was found among the products, the absence of the latter substance being an important factor in eliminating the possibility of nitrosobenzene as a primary product of oxidation. We are therefore led to believe that the first product of oxidation is phenylhydroxylamine and that the course of the reaction is as follows. Phenylhydroxylamine immediately couples with unchanged aniline to give 4-aminodiphenylamine and this on oxidation gives quinone phenyldiimide, I. I then couples with the 4-aminodiphenylamine present to give quinone-anil-(4 (4-amino-anilino) anil), II. This in turn undergoes further oxidation through emeraldine to nigraniline and the latter combines with unchanged aniline to give ungreenable aniline black in the manner suggested by Green and Wolff (1913).

$$C_6H_5NH_2 \longrightarrow C_6H_5NHOH \xrightarrow{+\,C_6H_5NH_2} C_6H_5NH\,.\,C_6H_4\,.\,NH_2 \longrightarrow$$



I II

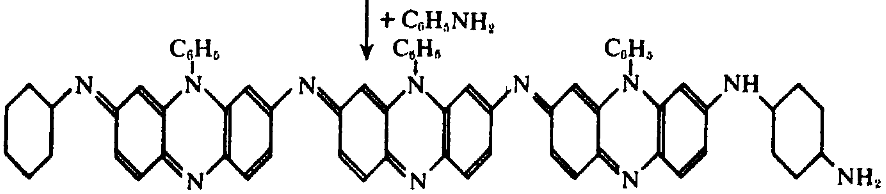
$$\longrightarrow C_6H_5N:C_6H_4:N\,.\,C_6H_4\,.\,N:C_6H_4:N\,.\,C_6H_4\,.\,NHC_6H_4\,.\,NH\,.\,C_6H_4NH\;C_6H_4NH_2$$

(Emeraldine)

$$\downarrow +O$$

$$C_6H_5N:C_6H_4:N\,.\,C_6H_4\,.\,N:C_6H_4:N\,.\,C_6H_4\,.\,N:C_6H_4:N\,.\,C_6H_4\,.\,NH\,.\,C_6H_4NH_2$$

(Nigraniline)

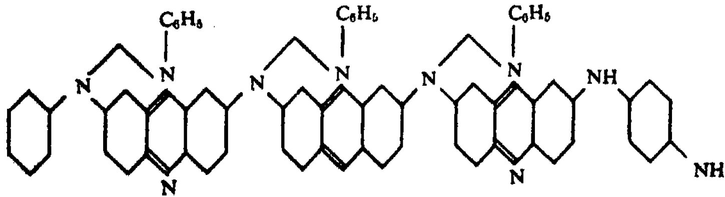


(Ungreenable aniline black)*

Support is given to the above scheme by the fact that peroxidase and dilute hydrogen peroxide react readily with 4-aminodiphenylamine in

---

* This formula for aniline black has not previously been put forward, but, for the reasons given below, it appears to be a logical modification of the classical formula established by Green:



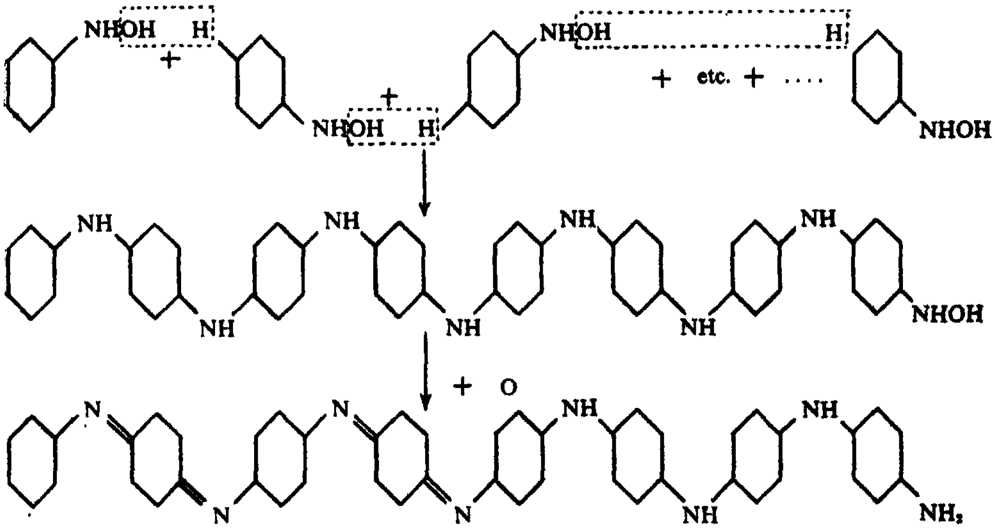
(*a*) Five membered ring systems of the type shown are highly improbable on stereochemical grounds.

(*b*) Modern electronic theories of valency rule out the possibility of five covalent linkages attached to the pentavalent nitrogen atom.

dilute acetic acid solution giving first a deep blue solution and then emeraldine which separates out as a deep green precipitate in theoretical yield. The blue colour is due undoubtedly to the imide II. The formation of this compound from 4-aminodiphenylamine and hydrogen peroxide does actually take place in the absence of the enzyme (Willstätter and Moore, 1907). It is an unstable compound and, as might be expected, the enzyme takes the oxidation a stage farther. The oxidation of 4-aminodiphenylamine to emeraldine using peroxidase is of interest in that it is analogous to the observation of Green and Woodhead (1910) who carried out the reaction using ferrous sulphate as the catalyst.

The enzyme was also found to be capable of oxidizing 4-aminodiphenylamine to emeraldine base in neutral solution, but in this case no blue imide was formed as an intermediate product, the solution becoming yellow and then yellowish red.

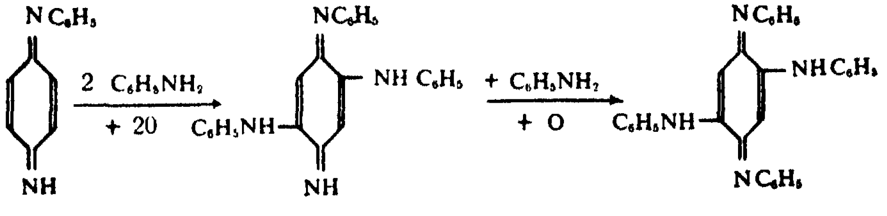
We were also able to show that the enzyme system was capable of oxidizing phenylhydroxylamine dissolved in dilute acetic acid directly to emeraldine. No transient blue-violet colour was, however, produced in this case. It seems probable, therefore, that eight phenylhydroxylamine molecules first condense together and that oxidation takes place subsequently.



We must therefore not overlook the fact that, in the enzymatic oxidation of aniline, the phenylhydroxylamine first formed may be oxidized directly to emeraldine. The presence of aniline may well, however, alter the course of the reaction and give rise to 4-aminodiphenylamine as an intermediate product as suggested above.

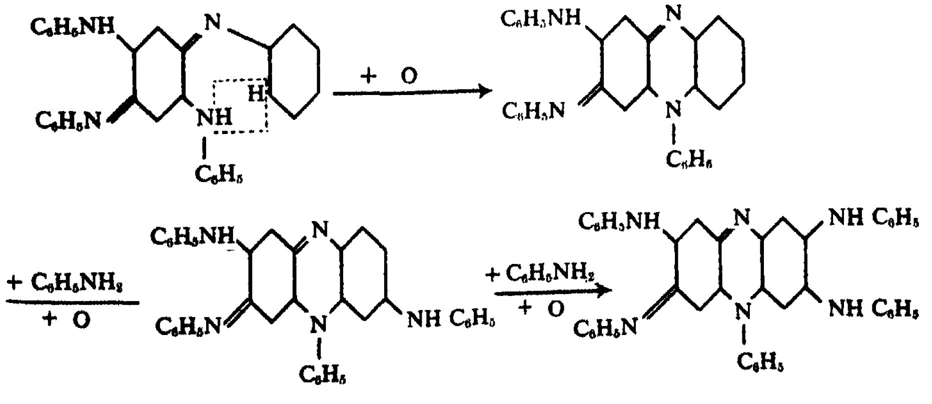
It is evident that quinone phenyldiimide can also react in another manner with aniline and the enzyme system which is distinct from the above. It is possible for aniline to react with the nuclear hydrogen atoms of quinone phenyldiimide to give 2: 5-dianilino-quinone-imide-anil, III. This substance was in fact isolated from the oxidation product in a yield of over 30%.

In order to account for the production of induline (*see* below), it is assumed that 2: 5-dianilino-quinone-imide-anil reacts with a further molecule of aniline to give azophenine IV (2: 5-dianilino-quinone-dianil), although this substance was not actually found among the products of oxidation:



III IV

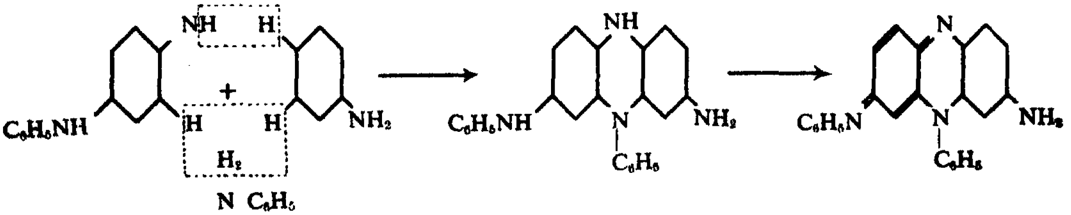
Witt and Thomas (1883) have previously shown that azophenine is an intermediate product in the formation of induline from aminoazobenzene and aniline. In the present instance, then, it is highly probable that the azophenine reacts immediately with aniline and the enzyme system to give indulines as follows:



Induline 3B Induline 6B

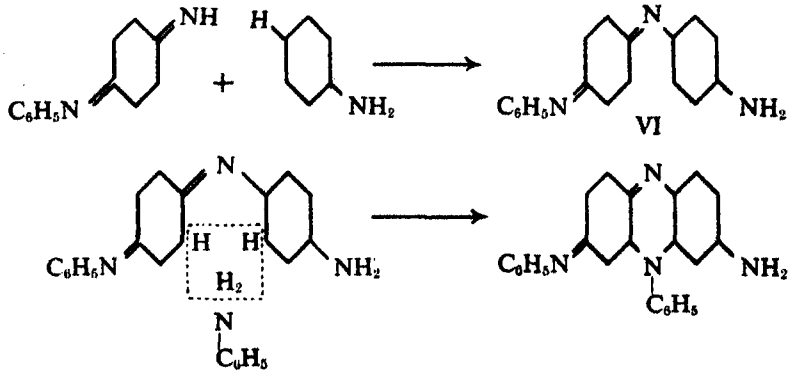
The substance which we obtained had properties which agreed very closely with those of induline 3B, but small quantities of another induline, probably induline 6B, appeared to contaminate the product. No doubt the actual induline formed depends upon the time of oxidation and the concentration as it does in the purely chemical methods of preparing these substances.

It is interesting to note that among our products we obtained pseudo-mauveine identical with that obtained by Perkin (1879) by oxidizing aniline with potassium dichromate. It may be that the production of this safranine (2-amino-8-anil-N-phenyl diphenazine, V) is effected by the oxidation of a mixture of one molecule of 4-aminodiphenylamine and two molecules of aniline thus:



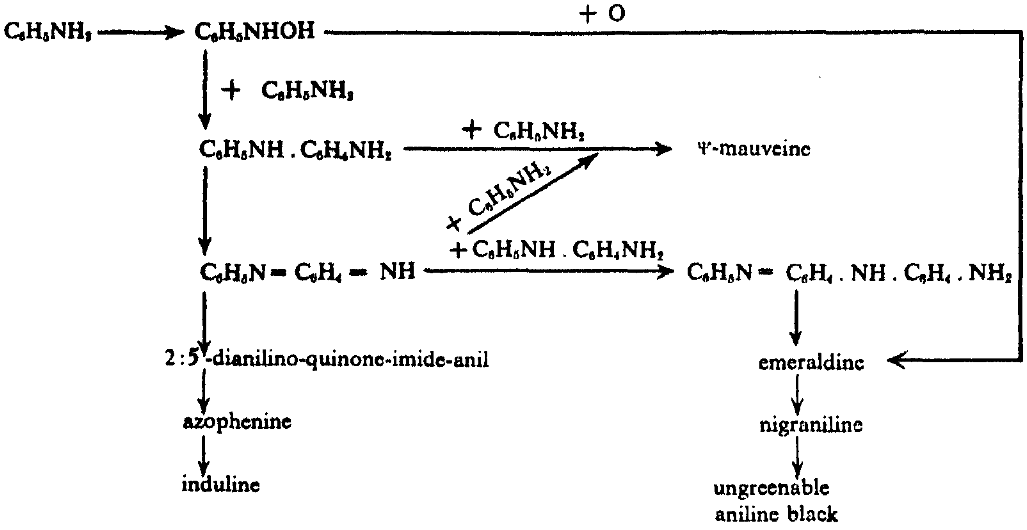
V Leuco-compound

It would probably be equally true to assume that quinone phenyldiimide reacts with one molecule of aniline to form an indamine VI which then condenses with a second molecule of aniline to give pseudo-mauveine:



On account of the facile oxidation of 4-aminodiphenylamine to quinone phenyldiimide the second explanation is probably more acceptable.

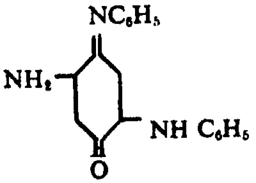
The production of 2: 5-dianilino-quinone-imide-anil, induline, ψ-mauveine and aniline black in one and the same reaction affords excellent confirmation of the generic relationships of these substances suggested from time to time by Willstätter and other workers. The formation of these substances considered in conjunction with the fact that the enzyme system has been found capable of oxidizing both phenylhydroxylamine and 4-aminodiphenylamine to emeraldine indicates that the enzymatic oxidation of aniline most probably proceeds according to the following scheme:



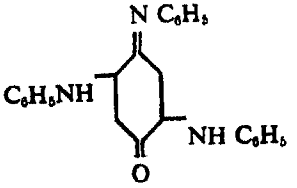
It is interesting to note that whereas purely chemical oxidations of aniline usually give rise to one or two products, the enzymatic oxidation gives rise to at least four. Azobenzene is frequently one of the oxidation products, *e.g.*, Börnstein (1901), Goldschmidt (1920), but its absence in the present instance suggests that the coupling of phenylhydroxylamine and aniline is extremely rapid and occurs before the further oxidation of the phenylhydroxylamine can take place.

The purely chemical oxidation of aniline by hydrogen peroxide has been recorded by different workers. Using dilute hydrogen peroxide, Bamberger and Tschirner (1898) obtained azobenzene, azoxybenzene, and nitrobenzene. By gently warming aniline acetate with hydrogen peroxide, Leeds (1881) obtained azobenzene. On boiling a solution of aniline in acetic acid with hydrogen peroxide for a long time, Schunck and Marchlewski (1892) obtained azobenzene and 2: 5-dianilino-benzo-quinone monoanil.

It is obviously of interest to determine to what extent the action of the enzyme can be imitated by the substitution of inorganic catalysts. We accordingly carried out the oxidation of aniline in acetic acid solution, using dilute hydrogen peroxide and ferrous sulphate (Fenton's reagent). No transient blue-violet colour was produced and a brown precipitate gradually separated which was shown to consist of azobenzene, amino-anilino-quinone-monoanil, VII, and 2: 5-dianilino-benzo-quinone monoanil, VIII. The absence of polycyclic products such as ψ-mauveine, induline, and aniline black is of interest. It is obvious, therefore, that



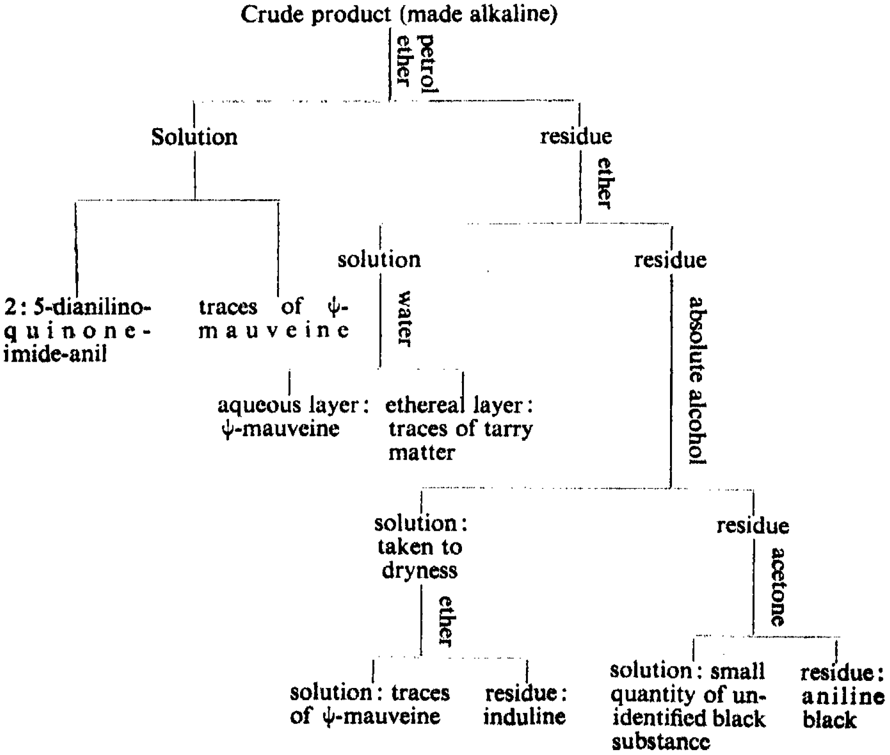
VII



VIII

Fenton's reagent behaves very differently from the enzyme system.

The isolation of the various products of the enzymatic oxidation of aniline was accomplished according to the scheme outlined below, details of which are given in the Experimental Part.


Crude product (made alkaline)
petrol ether
Solution
residue
ether
2:5-dianilinoquinone-imide-anil
traces of ψ-mauveine
solution
residue
water
absolute alcohol
aqueous layer: ψ-mauveine
ethereal layer: traces of tarry matter
solution: taken to dryness
residue
ether
acetone
solution: traces of ψ-mauveine
residue: induline
solution: small quantity of unidentified black substance
residue: aniline black

## EXPERIMENTAL

10 gm of aniline were dissolved in 10 cc of glacial acetic acid and diluted to a volume of 500 cc giving a solution of $p_H$ 4·5. To this solution were

added 3 cc of 10 volume hydrogen peroxide and a suitable amount of the enzyme preparation (20 mg of a preparation of purpurogallin number 100) dissolved in a few cubic centimetres of water. A blue-violet colour was first produced which rapidly changed to brown. This change in colour provides, in the early stages of the reaction, an indication of the necessity of a further addition of hydrogen peroxide, which was subsequently added 2 cc at a time. The blue-violet colour was seen after each subsequent addition of hydrogen peroxide until its formation was masked by the accumulation of the brown product. Addition of the peroxide was continued over a period of 12 to 24 hours, during which time a total of about 60 cc of 10 volume hydrogen peroxide were added and two further additions each of 10 mg of the enzyme preparation were also made. A faint odour of isocyanide was noticeable as the experiment proceeded. At the conclusion of the reaction the brown solid was filtered off. The filtrate was made alkaline by the addition of sodium hydroxide solution whereby a further small quantity of the bases of the same substances was obtained.

A quantity of the crude solid filtered off from the acid solution was subjected to steam distillation for a considerable time; neither azobenzene nor benzoquinone could be detected in the distillate. The precipitate obtained from the alkaline solution was also steam distilled: the distillate contained only a small quantity of unchanged aniline. In subsequent experiments the entire product at the conclusion of the reaction was made alkaline with sodium hydroxide, steam distilled and the residue dried and filtered. The yield varied between 5 and 7 gm.

2: 5-*dianilino-benzoquinone-imide-anil*—The separation of this substance is based on the fact that it is slightly soluble in petroleum ether whereas the other products are insoluble except ψ-mauveine which is only very sparingly soluble.

20 gm of the dried crude product were extracted in a soxhlet with 60–80° light petroleum until no more colour was extracted. This took several days. The extracts were reddish in colour, fresh petroleum being used about every 6 hours. The earlier extracts on being cooled deposited reddish brown crystals of m.p. 120-140°, and the filtrate from these on being concentrated gave further crops of crystals melting at about 120°. The later extracts deposited reddish violet crystals melting at 85–95°.

The fractions melting between 120° and 140° were combined and dissolved in hot methyl alcohol, water added until just cloudy and warmed to make clear. On standing, bright red crystals of 2: 5-dianilino-benzoquinone-imide-anil separated out, m.p. 167°. Mixed melting point with substance obtained according to directions of Majima and Aoki (1911) = 167°.

Found C = 79·03, H = 5·4, N = 15·7%.

$C_{24}H_{20}N_4$ requires C = 79·09, H = 5·54, N = 15·38%.

When treated with concentrated sulphuric acid it gave a reddish colour which turned deep blue on being heated and gave a blue-red fluorescent solution on dilution. This reaction is characteristic of 2: 5-dianilinoquinone-imide-anil and azophenine (Börnstein, 1901).

The identity of the substance was further confirmed by converting it into the acetyl derivative with acetic anhydride. Recrystallized from alcohol; m.p. 212°.

Found C = 77·03, H = 5·19, N = 13·72%.

$C_{26}H_{22}N_4O$ requires C = 77·23, H = 5·44, N = 13·79%.

It also gave azophenine when heated with aniline and acetic acid. Recrystallized from toluene; m.p. 236°. Börnstein (1901) gives 237°.

Found N = 12·52%. $C_{30}H_{24}N_4$ requires N = 12·72%.

The later fractions of m.p. 85–95° were difficult to purify owing to adsorption of small quantities of ψ-mauveine. The violet colour of the solid was evidently due to this. That it was essentially 2: 5-dianilinoquinone-imide-anil was shown by its intense colour reaction with sulphuric acid and by the fact that it gave good yields of the pure acetyl derivative and of azophenine. The presence of the safranine was demonstrated by dissolving the solid in alcohol, adding water until no more precipitate was produced and filtering. The aqueous alcoholic filtrate on being evaporated to dryness gave a green colour with concentrated sulphuric acid which became reddish violet on dilution. By repeating this process many times and then recrystallizing the solid from methyl alcohol as described above, the pure imide-anil was ultimately obtained.

*ψ-mauveine* (*phenomauveine or phenylphenosafranine*)—This component can be separated by making use of its slight solubility in ether. This solvent, however, also extracts a little tarry matter, but on shaking with water only the ψ-mauveine passes into the aqueous layer.

The residue after the petrol ether treatment was therefore extracted with ether for some considerable time in a soxhlet until no more red colour was removed. The ethereal solution was shaken twice with an equal volume of water and the aqueous extract, which contained nothing but ψ-mauveine, was evaporated to a small bulk under reduced pressure and the separated ψ-mauveine filtered off as a violet-black crystalline powder. With concentrated sulphuric acid it gave a deep green colour which turned red on dilution. It dissolved in alcohol to give a red solution, but without any yellow fluorescence, thus distinguishing it from phenosafranine which is theoretically a possible product of the reaction. The properties of the substance were found to be identical in every respect with those of an authentic specimen of ψ-mauveine prepared according to the directions of Perkin (1879).

Found N = 15·9. $C_{24}H_{20}N_4$ requires N = 15·4%.

The ethereal layer was found to contain only a small amount of tarry residue.

*Induline*—The residue after treatment with ether was extracted in a soxhlet with absolute alcohol until no more colour was removed. The deep blue solution which contained induline and traces of ψ-mauveine (which could never be completely removed by the previous extraction) was evaporated to dryness and extracted with ether to remove the ψ-mauveine. The residue was dissolved in hot benzene which on standing deposited minute blue-black crystals. These melted at 240°, but usually a trace of substance remained which did not melt finally until about 280°. Kehrmann and Klopstein (1923) give melting point of induline 3B as 242° and of 6B as 286–288°.

Found N = 13·3%. $C_{36}H_{27}N_5$ (induline 3B) requires N = 13·23%.

The substance had the properties ascribed to induline 3B by the above authors. In particular it dissolved in concentrated sulphuric acid with a blue-violet colour and on the addition of water the deep blue sulphate separated out.

*Ungreenable Aniline Black*—The residue left after the alcohol extraction was then extracted exhaustively with acetone. The acetone contained traces of residual induline and a small quantity of a black substance of indefinite composition. The residue after this extraction was a black powder from which benzene and toluene extracted nothing. It was shown to be ungreenable aniline black and was purified by dissolving it in 80% acetic acid, filtering and precipitating with dilute HCl. The precipitate was made alkaline with ammonia and thoroughly washed with water. After being dried at 120° it gave the following analysis:

Found C = 77·6, H = 4·63, N = 14·9%.

$C_{66}H_{45}N_{11} . H_2O$ (Green's formula) requires C = 78·49, H = 4·65, N = 15·26%.

The purified product dissolved in 80% acetic acid giving a dark greenish blue-black solution and possessed other properties similar to those of a specimen of ungreenable aniline black synthesized from nigraniline and aniline according to the method of Green and Wolff (1913).

### *Oxidation of 4-aminodiphenylamine by Peroxidase*

1 gm of 4-aminodiphenylamine was dissolved in 20 cc of glacial acetic acid and diluted to about 100 cc with water. 0·5 cc of 1·6 volume $H_2O_2$ was added and then a few drops of the enzyme solution. A deep blue colour was produced immediately which gradually intensified. Further quantities of 1 cc of $H_2O_2$ were added at intervals of about half an hour over a period of 8 hours. A few milligrams of enzyme were also added periodically. After a time the blue colour gradually disappeared and a green precipitate of emeraldine was produced. This was filtered off, dissolved in 80% acetic acid, and filtered. Dilute HCl was added and the precipitate so obtained was made alkaline with ammonia, filtered and washed. It was dried at 120° and gave N = 15·1%, $C_{48}H_{38}N_8$ requires N = 15·5%.

It dissolved in 80% acetic acid giving a bright green colour.

### *Oxidation of Phenylhydroxylamine by Peroxidase*

A small quantity of freshly prepared phenylhydroxylamine was dissolved in a little dilute acetic acid. A few drops of dilute hydrogen peroxide were added and then a few milligrams of enzyme. A deep green precipitate of emeraldine was produced immediately. Further quantities of hydrogen peroxide and enzyme were added at intervals and the emeraldine filtered off and identified as above.

### *The Oxidation of Aniline by Means of Fenton's Reagent*

10 gm of aniline were dissolved in 12 cc of glacial acetic acid and diluted with 100 cc of water. A few drops of 5% ferrous sulphate solution were added and then 1 cc of 20 volume hydrogen peroxide. The solution became brown, no transient blue or violet colour being observed. Further quantities of 1 cc of hydrogen peroxide were added at intervals over a period of 8 hours, a total of about 70 cc being added altogether. Further quantities of 0·5 cc of ferrous sulphate solution were also added about every hour. If too much ferrous sulphate was added at a time a tarry product was obtained which possessed a marked odour of isocyanide. At the end of about 12 hours the brown precipitate which had separated out was filtered off, washed, and steam distilled. Azobenzene crystallized out in the aqueous distillate and was filtered off and identified; m.p. 68°. Mixed melting point with an authentic specimen = 68°.

The residue from the steam distillation was filtered off, dried, and extracted with 60–80° petrol ether in a soxhlet until no more red colour was extracted. On concentrating and cooling the extract small red crystals separated out which were recrystallized from 60–80° petrol ether; m.p. 126°. The substance proved to be amino-anilino-quinone-monoanil for which Majima (1911) gives m.p. 127–128°. It dissolved in concentrated sulphuric acid to give a green colour which changed only slightly on being heated. It dissolved in 30% sulphuric acid to give a bright green solution which on being heated turned a brilliant purple.

Found C = 74·8, H = 5·10%.

$C_{18}H_{15}ON_3$ requires C = 74·71, H = 5·23%.

On being heated with aniline in glacial acetic acid solution it gave dianilino-quinone-monoanil; m.p. 202°.

The residue after the petrol ether extraction was extracted with alcohol in a soxhlet. On cooling the extract crystals of dianilino-quinone-monoanil were obtained; m.p. 202°. Found N = 11·6%. Dianilino-quinone-monoanil $C_{24}H_{19}ON_3$ requires N = 11·5%. The substance gave a characteristic blood red colour with concentrated sulphuric acid.

The residue after the alcohol extraction contained iron and was found to be insoluble in all organic solvents.

One of us (B. C. S.) is indebted to the Chemical Society for a grant.

## SUMMARY

The enzyme peroxidase derived either from horseradish or turnips readily oxidizes aniline in the presence of dilute hydrogen peroxide at $p_H$ 4·5 (dilute acetic acid solution).

During the first few seconds of the reaction a blue-violet coloration is produced which almost immediately turns brown. A dark brown precipitate is ultimately obtained which has been shown to consist of 2: 5-dianilino-quinone-imide-anil, ψ-mauveine, induline, and ungreenable aniline black.

A scheme suggesting the mechanism of the reaction is given in which phenylhydroxylamine is the primary product of oxidation and 4-aminodiphenylamine is an important precursor of the above substances.

The following experiments which have been carried out confirm the suggested mechanism:

(*a*) The enzyme system oxidizes freshly prepared phenylhydroxylamine spontaneously to emeraldine.

(*b*) The enzyme system oxidizes 4-aminodiphenylamine first to Willstätter's blue imide (quinone-anil-(4 (4-amino-anilino) anil) ) and then to emeraldine.

The action of Fenton's reagent (hydrogen peroxide and ferrous sulphate) on aniline dissolved in dilute acetic acid is entirely different from that of the enzyme, the products being azobenzene, amino-anilino-quinone-monoanil and 2 : 5-dianilino-quinone-monoanil.

## REFERENCES

Bach (1904). 'Ber. deuts. chem. Ges.,' vol. **37**, p. 3787.
Bamberger and Tschirner (1898). 'Ber. deuts. chem. Ges.,' vol. **31**, p. 1523.
Bertrand (1903). 'C.R. Acad. Sci., Paris,' vol. **134**, p. 124.
Börnstein (1901). 'Ber. deuts. chem. Ges.,' vol. **34**, p. 1280.
Bouquelot (1896). 'C.R. Acad. Sci., Paris,' vol. **123**, p. 315.
Chodat (1925). 'Handb. biol. ArbMeth.,' (4), vol. **1**, p. 319.
Elliott (1932, *a*). 'Biochem. J.,' vol. **26**, p. 10.
—— (1932, *b*). 'Biochem. J.,' vol. **26**, p. 128.
Goldschmidt (1920). 'Ber. deuts. chem. Ges.,' vol. **53**, p. 28.
Green and Woodhead (1910). 'J. Chem. Soc.,' vol. **97**, p. 2392.
—— and Wolff (1913). 'Ber. deuts. chem. Ges.,' vol. **46**, p. 33.
Kehrmann and Klopstein (1923). 'Ber. deuts. chem. Ges.,' vol. **56**, p. 2394.
Leeds (1881). 'Ber. deuts. chem. Ges.,' vol. **14**, p. 1384.
Majima (1911). 'Ber. deuts. chem. Ges.,' vol. **44**, p. 229.
Majima and Aoki (1911). 'Ber. deuts. chem. Ges.,' vol. **44**, p. 3080.
Perkin (1879). 'J. Chem. Soc.,' vol. **35**, p. 717.
Schunck and Marchlewski (1892). 'Ber. deuts. chem. Ges.,' vol. **25**, p. 3574.
Willstätter and Heiss (1923). 'Liebig's Ann.,' vol. **433**, p. 17.
—— and Moore (1907). 'Ber. deuts. chem. Ges.,' vol. **40**, p. 2665.
—— and Stoll (1918). 'Liebig's Ann.,' vol. **416**, p. 21.
Witt and Thomas (1883). 'J. Chem. Soc.,' vol. **43**, p. 112.

576 . 353

# The Effects of X-Rays on Mitosis in the Spermatogonial Divisions of *Locusta migratoria* L.

By M. J. D. WHITE, University College, London

(*Communicated by J. B. S. Haldane, F.R.S.—Received September* 2, 1935)

## 1—INTRODUCTION

Although the fact that X-rays cause major structural alterations in chromosomes was actually known long before the discovery of their capacity for increasing gene mutation-rates our knowledge of the former type of effect has lagged behind. Nearly all the recent work on the biological effects of X-rays has been carried out on material such as vertebrate tissue-cultures which are extremely unfavourable for detailed study of chromosomes. Hence the very vague conceptions of the influence of radiation on the nucleus which are widespread in the literature; many authors referring to the sum total of these effects as the " sensitivity " of the nucleus to radiation.

The literature dealing specifically with chromosomal abnormalities resulting from irradiation comprises only about nine papers (Helwig (1933); Huskins and Hunter (1935); Lewitsky and Araratian (1931); Mather and Stone (1934); Mather (1934); Navashin (1931); Stadler (1931); Stone (1933); and White (1935) ). Of course, a great deal of work has been done on translocations and inversions produced by X-rays (*e.g.*, Muller and Altenburg (1930): Muller, Prokofieva, and Raffel (1935) ), but this only refers to cytologically and genetically viable abnormalities.* It is obvious that these comprise only a small fraction of the total range of aberrations produced; the present paper, like those mentioned above, aims at recording *all* the artificially induced chromosome forms, whether capable of survival or not.

## 2—MATERIAL AND METHODS

In the experiments described below only newly emerged male imagines were used. A uniform dosage was applied, namely, Coolidge universal tube; unscreened radiation; 30 cm target distance; 5 milliamperes; 65 kilovolts; 7 minutes exposure.

---

* A cytologically viable abnormality is one capable of persisting through a number of cell divisions without change; a genetically viable one is one which is capable of existing in a zygote and giving rise to an organism all the cells of which contain the abnormality in question. The latter concept thus includes the former.

The animals were killed and their testes fixed in strong Flemming solution at varying times after exposure, namely, 12, 17, 24, 28, 32, 51 hours, and 3, 5, 6, 8, 9, 10, 11, 12, 13, and 14 days.

The methods of staining used were as described in a former paper (White, 1934, *b*).

The drawings were made at a uniform magnification of approximately × 5000, but they have been reduced in reproduction and the magnification is given below each figure. In a number of figures some of the chromosomes have been separated in drawing to eliminate overlapping as far as possible.

Certain difficulties inherent in this type of work may be pointed out here. In the first place the testes of insects contain cells in every conceivable stage of division, and it is consequently not possible, when looking at a nucleus in material fixed some hours or days after dosage, to tell at what stage of mitosis it was subjected to irradiation. The point is of great importance in view of the well-known fact that different stages of mitosis exhibit qualitatively as well as quantitatively different reactions to X-rays.

Another difficulty is that when observing the first division after irradiation (and to a less extent the second and subsequent divisions) the abnormalities are different in every cell; each observation is thus unique and cannot be verified by comparison with other nuclei. A judgement has thus to be made which is *probably* correct if the same type of abnormality has been met with elsewhere; but an identical aberration will never be met with again.

## 3—HISTOLOGY OF THE GONAD AND HISTOLOGICAL EFFECTS OF IRRADIATION

The testis of *Locusta* consists of a large number of digitiform follicles, each of which opens at its lower end into one of the two seminal ducts, the whole organ bearing a strong resemblance to a bunch of bananas. The histological structure of a follicle is shown in fig. 1, which represents a longitudinal section. The whole structure is solid, there being no lumen; near the tip of the follicle is the "apical cell" surrounded by the primordial spermatogonia; lower down are the lobules of secondary spermatogonia. As the primordial spermatogonia divide they give rise, first, to pairs of cells, then to quartets, octets, etc. The division of the cells in these groups is quite synchronous, and each group or lobule is

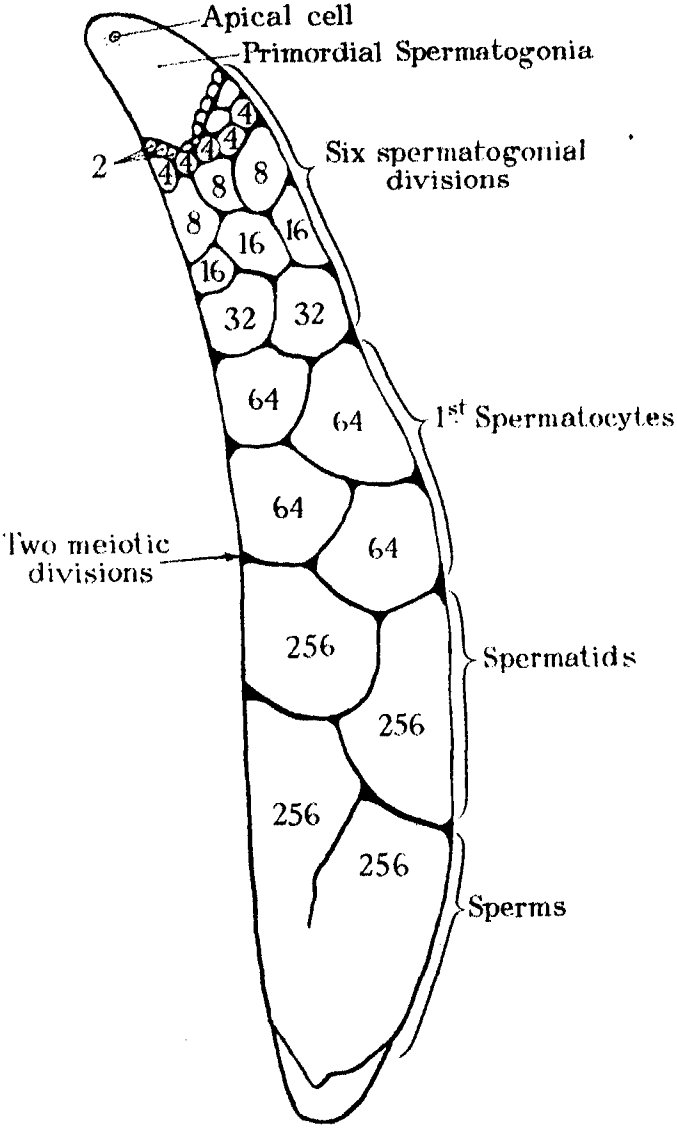


FIG. 1—Diagram of the histological arrangement of the lobules in a vertical section through a single testis-follicle. The numbers indicate the number of cells in each lobule. The two meiotic divisions follow on one another so rapidly that there is practically no interkinesis. Thus only in a follicle which shows metaphases of the second division will lobules containing 128 cells be found.

insulated from adjacent lobules by connective tissue. There are always six spermatogonial divisions, so that the lobules of first spermatocytes contain 64 cells.

It is not easy to determine the time taken by each of the spermatogonial divisions, but observations described later (§§ 7 and 8) suggest that about 48 hours are needed for the complete cycle from one metaphase to the next.

With the dosage employed a certain percentage of the spermatogonia is invariably killed. Those that are killed have pycnotic nuclei in which there is a single mass of chromatin of spherical or irregular shape which often later undergoes a secondary fragmentation into a number of spherules. These pycnotic nuclei are present, although rare, 12 hours after irradiation. Twenty-four hours after exposure there are great masses of them. At this stage normal metaphases are almost, but not quite, entirely absent. After some days fusion between adjacent pycnotic nuclei frequently occurs, giving rise to necrotic aggregates. It is quite usual for a whole lobule, consisting of 8 or 16 spermatogonia, to undergo pycnosis together. Pycnotic nuclei never pass into anaphase in this material, unlike those of irradiated Salamander epidermis (Alberti and Politzer, 1924).

These lethal effects are confined to the spermatogonia; the spermatocytes and the connective tissue cells between the lobules never show pycnosis. Of course, after a time (7–8 days) pycnotic nuclei and necrotic masses are found in and between the lobules of spermatocytes, but these are nuclei which were spermatogonia at the time of irradiation, and went into pycnosis at that time.

## 4—Normal Mitosis

The chromosomes in the spermatogonal divisions are 23 in number, 11 pairs of autosomes and the X-chromosome. Their metaphase-length and general appearance vary a great deal from one spermatogonial division to the next; at the metaphase of the first of the six divisions they are very long and thin, but the degree of metaphase-contraction increases with each successive division, so that at the last one they are very short and thick. The length of the long, medium, and short chromosomes at the last premeiotic division has been stated by me to be about 4·1 μ, 2·0 μ, and 1·0 μ, respectively (White, 1934); but at the first spermatogonial division they are at least 50% longer. This point is seen clearly if one compares figs. 5 or 10 with fig. 7.

Another point of difference between the early and the later spermatogonial divisions is the behaviour of the X-chromosome. In the early divisions (usually the first three) this never attains the degree of metaphase-contraction seen in the autosomes. When the nucleus as a whole is in late prophase the X-chromosome has only reached the degree of contraction ordinarily characteristic of early prophase, fig. 2; at metaphase, figs. 5, 6, 9, 10, 11, it is still in a state of contraction similar to that seen in the autosomes at mid-prophase; and even in anaphase, figs. 12, 13, 14,

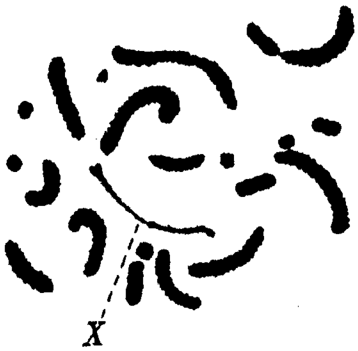


FIG. 2—Prophase chromosomes from 6-day material showing "negative heteropycnosis" of the X-chromosome. Not all the autosomes are drawn. × 3000.

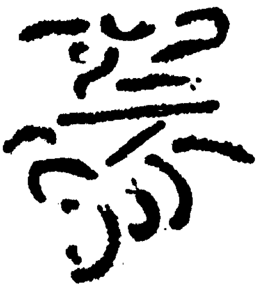


FIG. 3—Prophase chromosomes showing the already divided trabants in some cases. Not all the chromosomes drawn. 6-day material. × 3000.

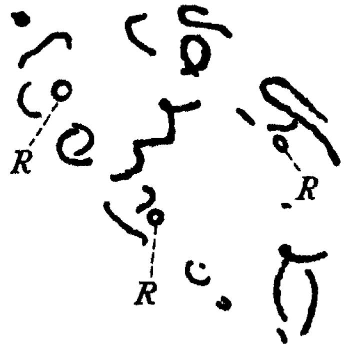


FIG. 4—Prophase from 5-day material showing three ring chromosomes. Not all the other chromosomes drawn. × 3000.

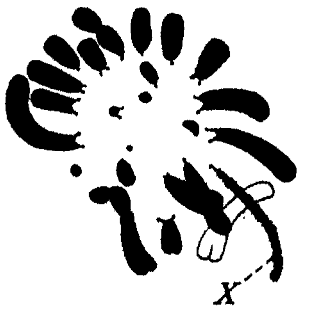


FIG. 5—Metaphase plate from 6-day material showing the negative heteropycnosis of the X. The trabants are well seen in most of the chromosomes. A single chromosome fragment is present (shown in outline). Several of the chromosomes show signs of having received translocations. × 3000.

it still has a slender appearance with an uneven outline. This behaviour of the X at the early spermatogonial divisions can be described as *negative heteropycnosis* to distinguish it from the *positive heteropycnosis* which occurs during the prophase of the first meiotic division (when the X reaches the metaphase degree of contraction long before the autosomes and remains in this state right up till pro-metaphase).

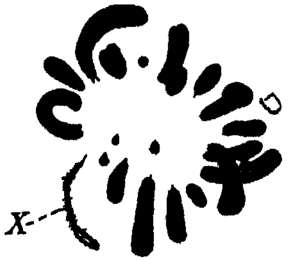


FIG. 6—Metaphase plate from 6-day material showing the negative heteropycnosis of the X, a single chromosome fragment (in outline) and a chromosome with two spindle attachments. × 3000.

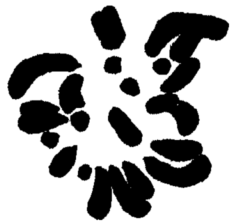


FIG. 7—A metaphase plate from one of the later spermatogonial divisions. X-chromosome not visible. × 3000.

A number of authors has illustrated this negative heteropycnosis in different species of *Acridiidae*; one gets the impression from studying their figures that they did not find it a very constant feature, which is so if one does not distinguish the early from the late spermatogonial divisions.

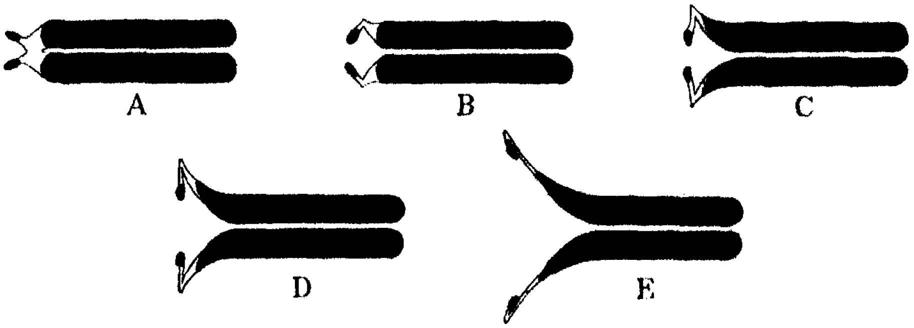


FIG. 8—A, prometaphase; B, metaphase ; and C–E, stages of anaphase-separation in a chromosome with " terminal " (really sub-terminal) spindle attachment.

The differential behaviour of the X is always present in the early divisions, however, both in the present species and in *Stenobothrus* spp. and hence probably throughout the Acridiidae. One effect of the negative heteropycnosis is that the split down the centre of each chromosome which is so obvious in mid-prophase, but which usually disappears from sight in the autosomes at metaphase owing to the thickening of the chromatids, is still clearly visible in the X-chromosome at metaphase. This is clearly seen in a number of the published figures (*e.g.*, Winiwarter, 1931, and Rao, 1934). Most frequently the split is obscured in the region immediately adjacent to the spindle attachment for a distance representing about one-sixth of the total length of the X-chromosome, figs. 9 and 11; frequently it is obscured at both ends, fig. 13, *a* and *b*, being quite clear in the middle. It appears that the degree of contraction is not quite uniform along the length of the chromosome, but is somewhat greater in the neighbourhood of the spindle attachment and sometimes also at the other end.

In material whose fixation has been mediocre no constrictions are observable in the spermatogonial chromosomes, and the same is in any case true of the chromosomes in the later spermatogonial divisions, however well fixed. In good early divisions, however, one can always see a constriction very near the proximal (spindle attachment) end of the chromosome. Beyond the constriction is a minute " trabant " or " polar granule " which is at or below the threshold of optical resolution. In each chromosome there are two such trabants, one for each chromatid; this is clearly to be seen at prophase, fig. 3, and also in slightly oblique views of metaphase plates, fig. 5. Usually, owing to unequal fixation, the trabants are not visible in all the chromosomes of a given nucleus, but in the most favourable cases they all show them.

Similar trabants have been figured by Janssens (1924) for other Acridiidae. They are similar to the trabant at the proximal end of the X-chromosome in *Drosophila melanogaster* (Kaufmann, 1934, and Prokofieva, 1935) and to the polar granules in the chromosomes of teleost fishes (Prokofieva, 1934) and in those of the rat (Koller and Darlington, 1934). In all these occurrences of chromosomes with " terminal " spindle attachments (" rod-shaped " chromosomes) it would appear that the attachment constriction is not quite at the end. In view of the difficulties of observation, it seems legitimate to suppose that all " rod-shaped " chromosomes have this fundamental structure. The question now arises: is it the constriction or the trabant which is the actual organ of attachment? Janssens (1924) believed it to be the latter; and Darlington (1933) has described " attachment chromomeres " in *Agapanthus* and *Kniphofia* which are similar to the terminal granules in the chromosomes of *Locusta*, except that the latter stain as deeply or deeper than the rest of the chromatid, while the former do not. Neither of these authors, however, has given any critical evidence to show that it is the chromomere rather than the constriction or constrictions associated with it which is the actual attachment point. However, the differential staining reactions of Darlington's " attachment chromomere " probably do indicate that it is chemically different from the chromomeres of the main chromatids.

It is believed that the present work has solved the problem of whether it is the constriction or the trabant which is the point of attachment, at any rate as far as *Locusta* is concerned, by a careful study of the critical stages of anaphase separation. The details are best seen in the X-chromosome, figs. 13, *a* and *b*, and 14, since the low degree of contraction renders

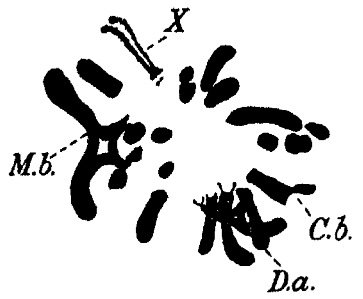


FIG. 9—Metaphase plate from 32-hour material. X-chromosome obvious. M.b. = mitotic bivalent (reciprocal chromatid fusion). C.b. = Chromatid break; D.a. = complex structure with two spindle attachments. × 3000.

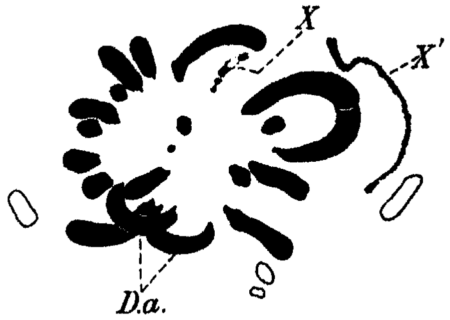


FIG. 10—Metaphase plate from 5-day material showing a broken X-chromosome. Four autosomal fragments drawn in outline only. × 3000.

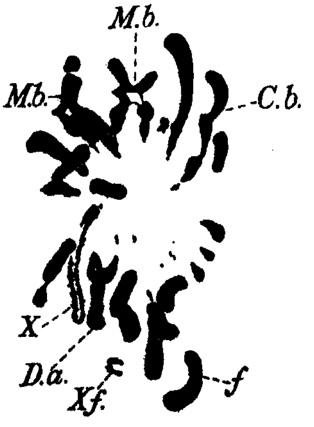


FIG. 11—Metaphase plate from 32-hour material showing a double chromatid break in the X. X.f. = fragment of the X lying outside the spindle region. Other lettering as in fig. 9. × 3000.

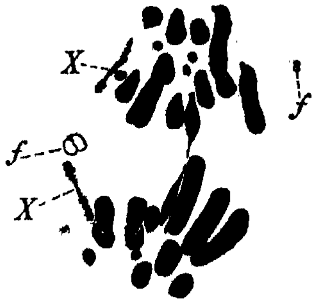


FIG. 12—Anaphase from 6-day material showing the X-chromosome. Fragments (in outline) dividing. × 3000.

the trabant and its constriction particularly clear; in the autosomes the higher degree of contraction seems to lead to a shortening of the constriction and a consequent drawing in of the trabant very close up to the body of the chromosome. Observations have, however, been made on anaphase separation in the autosomes which leave little doubt that the details are the same as in the sex-chromosome.

The first of the critical stages is illustrated in fig. 13, *a* and *b*. In both these X-chromosomes (from different cells, naturally) the trabants are seen as a pair of minute ovoid bodies in line with one another in the long axis of the spindle, *i.e.*, at right angles to the main body of the chromosome. This stage represents the very beginning of anaphase. The second critical stage occurs at the end of the first part of anaphase separation (just before the elongation of the middle portion of the spindle to form a Stemmkörper). This stage is illustrated in fig. 14; here the trabants are seen *alongside* the tip of the main chromatid (and not in line with it).

It is obvious that both these stages are irreconcilable with the theory that the " polar granule " is an " attachment chromomere." The details of anaphase separation must be as represented in fig. 8, and in the absence of any visible granule in the middle of the constriction (as in *Agapanthus* and *Kniphofia*) we must regard the non-staining gap between the main chromatid and the trabant as the actual organ of attachment to the spindle. It should be emphasized that the two " critical " stages are of short duration, and consequently rare in the preparations; between them there intervenes a much longer stage (shown in fig. 8, E, and in Janssens's paper of 1924), during which the trabants are *almost* in line with the tips of the main chromatids, so that one might suppose them to be the actual attachments.

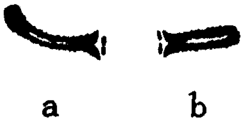


FIG. 13, *a* and *b*—X-chromosomes from two cells in late metaphase or very early anaphase. Note the position of the polar trabants. 51-hour material. × 3000.

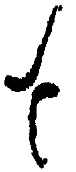


FIG. 14—X-chromosomes from an anaphase stage in 32-hour material. Note the polar trabants. × 3000.

If the trabants are not attachment-bodies, are they merely very short chromatids not differing save in size from the main chromatids on the other side of the constriction, or are they special bodies which, though not themselves points of attachment, nevertheless play an important part in the attachment-process? In highly de-stained preparations the main chromatids look faint and ghost-like; but the trabants remain minute dark points, the effect being just like that figured by Trankowsky (1930). This may indicate that the trabants have a different chemical constitution from that of the main chromatids, and it is consequently quite possible that their functions are also of a special nature.

## 5—MITOSIS UP TO 28 HOURS AFTER IRRADIATION

A small amount of material was available from four animals fixed 12, 17, 24, and 28 hours after irradiation. In the 12- and 17-hour individuals pycnosis was rare and metaphase plates relatively common, while in the 24- and 28-hour material the reverse occurred. A sufficient number of metaphases were observed, however, to show that there are no important differences between the chromosomal abnormalities at these four times after dosage. In fact, the only abnormalities met with were *chromosome breaks* (in which both chromatids have been broken at the same level) and translocations (possibly always reciprocal, but one cannot be sure). Both these are illustrated in fig. 15, which represents a metaphase plate from the 17-hour individual.

## 6—MITOSIS 32 HOURS AFTER IRRADIATION

Since the types of abnormalities found in the 32-hour material are very different from those encountered before (28 hours) or after (51 hours) it will be convenient to consider them separately.

Metaphase plates in the 32-hour material present many complex abnormalities, figs. 9 and 11. Since the individual chromatids are not distinguishable as in the work of Huskins and Hunter (1935), it is not possible to analyse the abnormalities completely in terms of the chromatids which are involved in them; but certain types stand out clearly. One of the commonest aberrations is the chromosome break, already met with. Chromosome breaks give rise to two kinds of fragments, those possessing and those without a spindle attachment; the former behave like whole chromosomes while the latter do not become attached to the spindle and lie right outside the metaphase plate, "*f*" in fig. 11. In the following account the term *fragment* will be used to signify a portion of a chromosome lacking a spindle attachment, the "distal fragments" of Mather and Stone. The average number of these fragments is 2·48 in 32-hour material.

Less frequently encountered are ***chromatid breaks*** in which only one chromatid is broken at a particular locus. These are not easy to observe in autosomes where the chromatids are not visible as separate threads at metaphase; but fig. 11 shows a clear case of two breaks in the same chromatid of the X-chromosome, the intervening portion, X*f*, having been ripped out lies well to one side of the metaphase plate. Such chromatid breaks were not found by Mather and Stone (1935), but

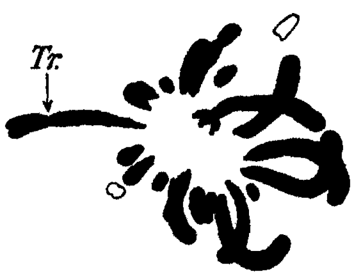


FIG. 15—Metaphase plate from 17-hour material showing chromosome fragments in outline. Tr. = a point of translocation (? to the X-chromosome). × 3000.

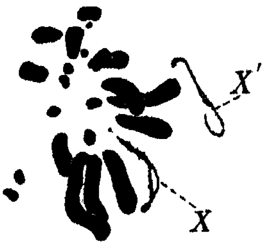


FIG. 16—Metaphase plate showing a whole X-chromosome attached to the spindle and a large X-fragment (X′) not attached. × 3000.

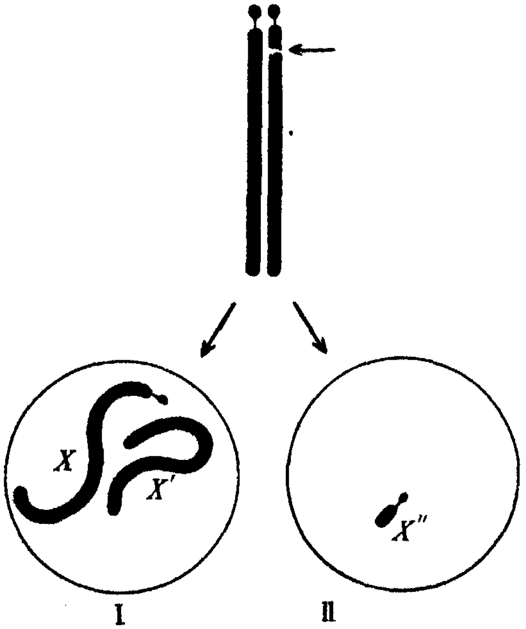


FIG. 17—Diagram showing a possible mode of origin of the cell shown in fig. 16.

occurred frequently in the work of Huskins and Hunter (1935); their significance will be discussed later in connexion with the problem of the time of division of the chromosomes in the mitotic cycle. The frequency of chromatid breaks in the present material is rather difficult to estimate, but complete breakage of the type seen in the X-chromosome of fig. 11 is rare; certain irregularities of chromosome outline, C.b., such as are to be seen in figs. 9 and 11, are, however, either chromatid breaks or examples of partial reattachment following on chromatid breaks.

Most frequent of all the abnormalities in 32-hour material are chromosomes with two points of attachment to the spindle, in which it is clear that fusion has taken place between two chromosomes. The structures labelled M b. and D.a. in figs. 9 and 11 are examples of this type of aberration. Some of them are difficult to analyse in terms of chromatids, but it is probable that all are *reciprocal chromatid fusions*. The clearest example is M.b. in fig. 9, represented diagrammatically in fig. 18. Here it is clear that a sort of somatic crossing over has taken place between two of the four chromatids at the level in question. This has resulted in a structure which may be described as a " mitotic bivalent." It differs from a true meiotic bivalent, fig. 19, in that one of the four chromatids has two spindle attachments, while one of the others has none. At the next division the latter will appear as a fragment; the chances of the chromatid with two attachments persisting to the next division without being broken into two will probably be half unless the distance between

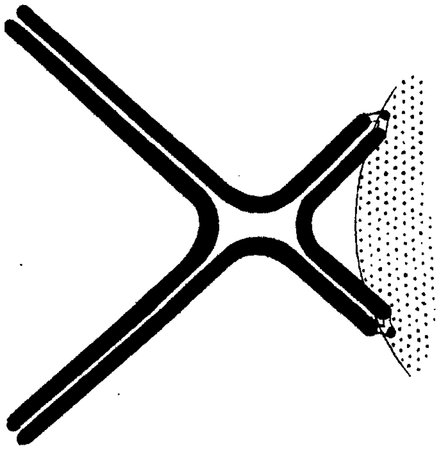


FIG. 18—Diagram of a " mitotic bivalent " (reciprocal chromatid fusion).

the two attachments is very short when, for mechanical reasons, one might expect it to be higher.

The structures labelled D.a. in figs. 9 and 11 are more difficult to interpret; it is clear that they have two attachments; but the chromatids are not sufficiently clear at the point of junction to permit complete analysis. However, it seems clear that " false " lateral fusions of the type represented in fig. 20, *b*, rather than " true " fusions as in fig. 20, *a*, are involved, and this is confirmed by a study of the next few divisions, in which no branched chromatids were found. The structures labelled M.b. in fig. 11 are probably " mitotic bivalents " of the same type as the clear case in fig. 9.

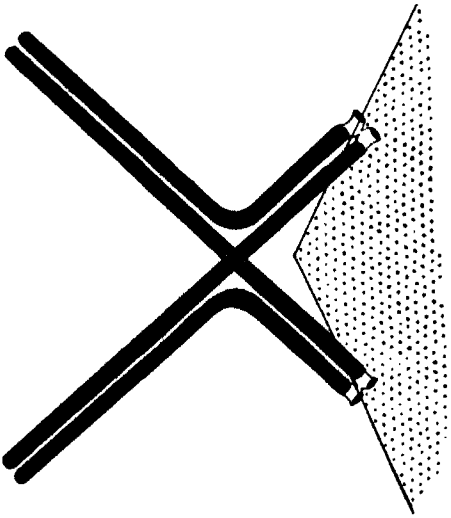


FIG. 19—Diagram of a meiotic bivalent with a single chiasma—to compare with fig. 18.

It is interesting to note that in the " mitotic bivalents " the spindle attachments of the two original chromosomes are in the same plane (*i.e.*, the equator of the spindle) at metaphase. On the other hand, we know that at meiosis the spindle attachments of a true bivalent arrange themselves above and below the equatorial plane, *i.e.*, in an axial direction. This point is made clear in figs. 18 and 19. In view of the fact that the spindle attachments all repel one another at metaphase, one can regard this difference as a proof that they divide at prometaphase of mitosis, while in meiosis they do not divide until the second division. Thus in the " mitotic bivalent " there are actually four attachments at the time of spindle-formation, which consequently arrange themselves with *one* *member of each pair* above and below the equatorial plane, just as in the case of the undivided attachments at meiosis.

## 7—MITOSIS MORE THAN 32 HOURS AFTER IRRADIATION

The abnormalities found in material fixed 51 hours and more after irradiation are very different from those met with in the 32-hour material, and resemble those in the 12–28 hour material much more closely. For reasons to be given later, it seems probable that only the 12–32-hour material represents the first division after irradiation, the later material containing second, third, and eventually fourth divisions.

Various abnormalities found in these divisions are illustrated in figs. 4, 5, 6, 10, 12, and 16. In addition, a novel type of chromosome resulting from irradiation is shown in figs. 22–25 and described in § 8.

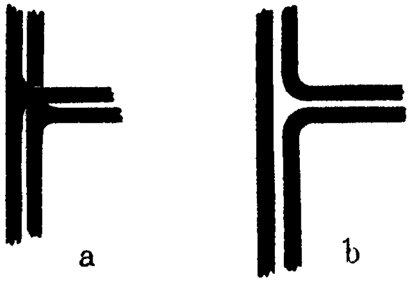


FIG. 20—Diagram showing the difference between a true (*a*) and false (*b*) lateral translocation.

As in the 32-hour material, chromosome breaks are common but become progressively rarer with time; thus in the 9-day material their frequency has decreased to 0·55 per cell, and by the 14th day it has fallen so low that it cannot be determined with the limited amount of material available. Some data on the number of fragments present at different times after irradiation are set out in Table I, and shown graphically in fig. 21. The fragments appear to divide quite normally, but sometimes separate tardily; compare those labelled "f" in fig. 12. Since they lack spindle attachments, there is no mechanism for their segregation and they are consequently distributed at random to the two daughter cells at cytokinesis. The progressive decrease in the number of fragments per nucleus from the first to the fourteenth day seems to result from re-attachment of fragments to chromosomes with spindle attachments. There is no evidence in this material of fragments being eliminated or absorbed in the cytoplasm; those seen on the ninth to the fourteenth days are just as chromophilic as in the 32-hour material.

Fragmentation of the X only occurs very rarely; figs. 10 and 16 represent the only undoubted cases—there are some others in which two or three X-fragments lie in close proximity to one another in a straight line, but with no visible connexion—these possibly represent reattachment of the X-chromosome following breakage. The subject of fragmentation of the X-chromosome is taken up later in § 10. Fig. 16 represents a particularly interesting case, since in addition to the very large X-fragment (X′) there is a whole X attached to the spindle. Such a condition probably arose through a chromatid break of the X-chromosome at a previous

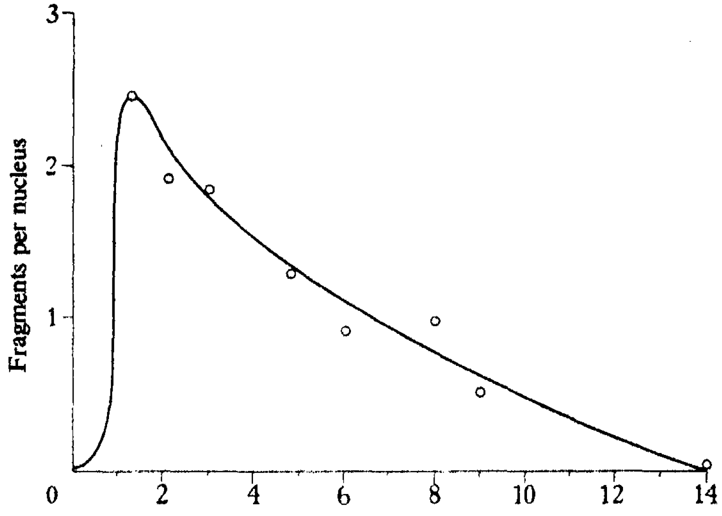


FIG. 21—Graph derived from the data in Table I, showing the number of fragments at different times after irradiation.

TABLE I—NUMBERS OF FRAGMENTS AT DIFFERENT TIMES AFTER IRRADIATION

| Time | Cells with 0, 1, 2, … fragments | | | | | | | | | | Average No. of fragments | Total cells |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | | |
| 32 hours …. | 11 | 13 | 8 | 4 | 5 | 4 | 4 | 1 | — | — | 2·48 | 50 |
| 51 ,, …. | 4 | 5 | 1 | 2 | 2 | 2 | — | — | — | — | 1·94 | 16 |
| 3 days …. | 20 | 8 | 6 | — | — | 1 | 1 | 1 | — | 1 | 1·86 | 38 |
| 5 ,, …. | 7 | 4 | 1 | 1 | 1 | — | — | 1 | — | — | 1·30 | 15 |
| 6 ,, …. | 8 | 3 | 2 | — | 4 | — | — | — | — | — | 0·93 | 19 |
| 8 ,, …. | 26 | 11 | 6 | 3 | 3 | — | 1 | — | — | — | 1·00 | 50 |
| 9 ,, …. | 16 | 7 | 4 | — | — | — | — | — | — | — | 0·55 | 27 |
| 14 ,, …. | 36 | 2 | — | — | — | — | — | — | — | — | 0·05 | 38 |

division, according to the diagram, fig. 17; but no cell of type II was found adjacent to it—perhaps one of the resting cells in the neighbourhood represents the missing nucleus.

Chromatid breaks and reciprocal chromatid fusions are entirely absent from material fixed 51 hours and more after irradiation; this is to be expected if the metaphases are of the second, third, etc., divisions after irradiation. On the other hand, chromosomes with two spindle attachments are still relatively common in 5- and 6-day material, D.a. in figs. 6 and 10; it is to be presumed that they arise from " mitotic bivalents " at the first division following irradiation, or from simple fusion between chromosomes, either between their original distal ends or between their new ends after loss of a fragment. No case of lateral translocation was observed in the 52-hour and later material.

Among the rarest abnormalities encountered are ring chromosomes. The scarcity of this type of aberration is in agreement with the findings of other workers on irradiated chromosomes (Mather and Stone found " two possible cases," Huskins and Hunter figure a single undoubted one). One point of interest in connexion with ring chromosomes is that there is a marked tendency for more than one ring to be present per cell; fig. 4, where there are three rings in one nucleus shows this state of affairs. Not all nuclei have more than one, however; in 3-day material only one ring per cell has been found; but in 5-day material two or three per cell is usual. This point is of significance, since less than 1% of cells show rings at all. Two explanations are possible, (1) that in certain nuclei irradiation sets up a particular physiological state in which the formation of rings is favoured, (2) that regular anaphase separation is difficult or impossible in rings, and that non-disjunction is consequently unusually frequent in them. The latter explanation is far more probable, in view of the observations of Navashin (1930) on interlocking of ring-chromosomes in *Crepis*.

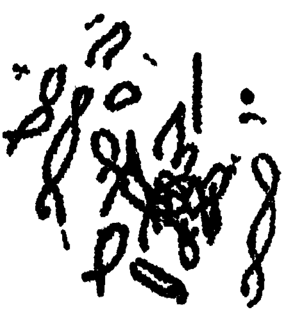


Fig. 22—Nucleus with diplochromosomes at early prophase. × 3000.

## 8—A New Chromosome Abnormality

An entirely new kind of chromosomal abnormality is represented in figs. 22–25 and interpreted in the diagram, fig. 26. A preliminary account of this type of abnormality has already appeared (White, 1935), but it seems desirable to describe it here in detail. Cells of this type have been

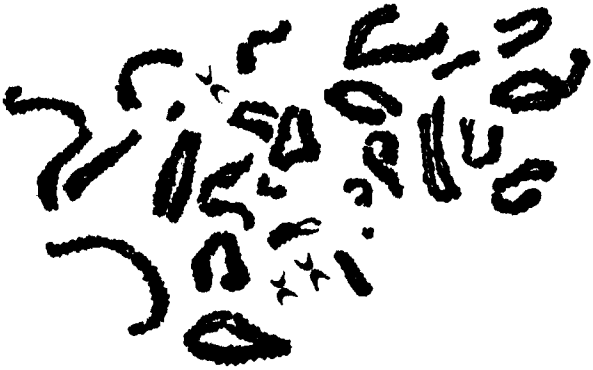


Fig. 23

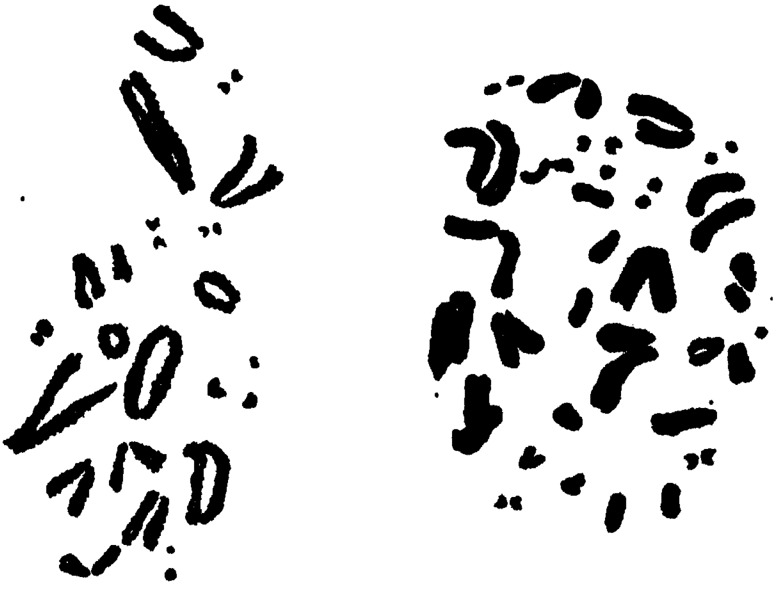


Fig. 24

Fig. 25

Figs. 23–25—Nuclei with diplochromosomes in middle and late prophase. × 3000.

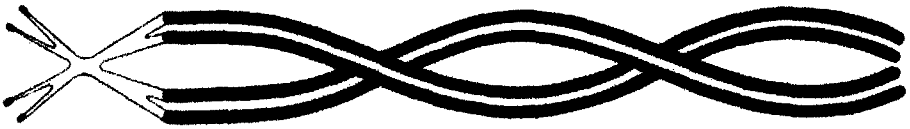


Fig. 26—Diagram of a diplochromosome in early prophase when the two limbs are still spirally wound round one another.

found in five different individual locusts, two fixed 6 days after irradiation, the others, 3, 8, and 11 days after. In each of these only a small percentage of the spermatogonial divisions (between 1 and 10%) were of this type. On examining the prophases, figs. 22–25, it will be seen that although the chromosomes are present in approximately the diploid number (actually there are a few fragments which make it appear slightly in excess of this) each chromosome is V-shaped, the limbs of the V being longitudinally split into two chromatids with, frequently, a very clear split between them. Sometimes the two arms of the V are slightly separated at the point of junction, so as to leave a non-staining gap—in other cases this is less distinct. A rough measurement shows that the length of these chromosomes is about twice the length of normal prophase chromosomes at approximately the same stage. Thus, although these nuclei are tetraploid in respect of the chromatids they only possess the diploid number of spindle attachments; each " chromosome " is a compound structure with two genetically homologous arms and a spindle attachment in the middle. Unfortunately, it has not been possible to demonstrate the presence of polar trabants in these chromosomes up till now; but they must be attached to the " median " spindle attachment, and are presumably four in number, like the long chromatids, fig. 26. The fact that they have not been seen yet is hardly surprising, in view of the great difficulty in making them out, even in normal chromosomes.

I shall refer to these structures as *diplochromosomes*, an ordinary chromosome of the type represented in fig. 8 being a *monochromosome*. Monochromosomes consist of four chromatids and a spindle attachment; in chromosomes which have hitherto been considered to have terminal spindle attachments two of these are very short and constitute the " polar granules." Diplochromosomes consist of eight chromatids (in the present case four long ones and four polar granules).

An interesting feature about the earliest prophase stage of the diplochromosomes is that the two limbs of the V are spirally wound round one another, fig. 22. This appears to be a constant and significant feature of the earliest stages; but it is completely lost by mid-prophase, probably as a result of the contraction which has taken place. The diagram, fig. 26, thus only represents an early prophase diplochromosome.

There are two possible ways in which diplochromosomes might be supposed to have arisen from normal chromosomes. According to the first hypothesis, two longitudinal divisions of the chromatids have taken place since the previous metaphase. The spindle-attachment, being undivided, holds the chromatids together. A situation analogous to that which normally occurs at pachytene has thus arisen—four homologous chromatids lying side by side. As at pachytene, these separate into a pair of paired chromatids.

The second hypothesis is that diplochromosomes result from an inhibition of the division of the spindle attachment which normally takes place at prometaphase. This leads to a failure of anaphase-separation at that division. Metaphase thus passes directly into a telophase in which partial uncoiling of the chromatids (which form two independent spirals) takes place; this partial uncoiling establishes a spiral relationship between the two homologous chromatids, *i.e.*, a state of affairs in which the two chromatids are coiled *round one another* as in a piece of electric flex. During the succeeding resting stage the normal chromatid " split " gives rise to a diplochromosome. The spiral relationship between the two arms of the V is still present in early prophase.

At present it does not appear to be possible to decide between the two hypotheses with certainty, but the relational spiral at early prophase speaks in favour of the second rather than the first hypothesis.

There is an obvious analogy between the structure of a diplochromosome and an " attached-X " chromosome in *Drosophila*. The mode of origin of the latter is by no means clear, but since they have arisen in the male (Sturtevant, 1931) it appears that in some instances, at least, they have been formed in the same way as diplochromosomes in *Locusta* (and not by a fusion between two originally separate chromosomes). It is interesting to find that a general nuclear reaction in one organism (*Locusta*) may affect only the sex chromosome in another (*Drosophila*).

Naturally most of the nuclei with diplochromosomes contain other types of abnormalities (chromosome breaks, etc.); but there are always 23 diplochromosomes unless fusion between two chromosomes has taken place, leading to diplochromosomes with two spindle attachments, one at each end. The latter appear as rings with two non-staining gaps. Thus in fig. 23 there are 19 V's and a couple of rings, together with a number of fragments which, since they have no spindle attachments, do not show the diplochromosome structure. In fig. 25 there are 23 diplochromosomes and some fragments, but no rings.

It is not certain whether the *Locusta* diplochromosomes are, like the attached-X, self-perpetuating structures, or whether, in the course of a few divisions, they eventually separate into two monochromosomes, thus giving rise eventually to a tetraploid nucleus of the normal type (White, 1934, *a*). In a number of plants (*e.g.*, *Spinacia* and some *Cruciferae*—Langlet (1927) and Manton (1935) ) and in some tumour cells (Ludford (1932) and Crew and Koller (1933) ) tetraploid cells have been figured in which the homologous chromosomes lie very close side by side. This appears to be a temporary phenomenon which disappears in the course of a few divisions; but it is not clear from the published figures whether these are really cases of " diplochromosomes " with a single spindle attachment for each pair of homologues.

In two cases groups of more than one cell with diplochromosomes were observed. The first consists of two cells in metaphase in 8-day material. Unfortunately, it was not possible to determine whether all the chromosomes had the structure of diplochromosomes or only some of them. Assuming the diplochromosome condition to have arisen at the division following irradiation, part of a mitotic cycle followed by two complete ones must have taken place in 8 days. In the second case a group of about 32 tetraploid cells were observed in 11-day material, some of which undoubtedly contained diplochromosomes (crowding of twice the usual number of chromosomes onto a metaphase plate very little if at all larger than normal makes it very difficult to tell whether one is dealing with chromosomes which are attached at the spindle attachment or not). Allowing for the fact that the earlier spermatogonial divisions are probably separated by longer " resting stages " than the later ones, we shall probably not be far wrong if we take 48 hours as the *average* length of the spermatogonial mitotic cycle.

## 9—FRAGMENTATION OF THE X-CHROMOSOME

The number of fragments seen in cells 32 hours after irradiation is relatively high (average 2·48 per cell). It is thus not possible to identify with any certainty the chromosomes from which they were derived in such a way as to be able to compare the frequency of fragmentation in the different chromosomes. Owing, however, to the fact that in the first three spermatogonial divisions the X is easily recognizable by its negative heteropycnosis it is possible to compare it with the autosomes in respect of fragmentation-frequency (it should be pointed out that X-fragments, even when separated from the main part of the X, still retain their original degree of contraction unchanged). If fragmentation occurred equally in all the chromosomes we should expect to find the X broken in 2·48/23 or in about one-tenth of the cells observed in metaphase 32 hours after irradiation. This is assuming the frequency of fragmentation to be independent of the length of the chromosome. Actually the X is one of the longer chromosomes, so that if allowance is made for this fact we should expect it to be broken in rather more than a tenth. In fact, it is only fragmented in about one cell per hundred examined—figs. 10, 11, and 16 represent the only undoubted cases seen in the whole investigation;

the existence of some doubtful cases (referred to above in § 7) makes it advisable not to give a more definite estimate of the frequency of fragmentation in the X—but 1 in 100 represents the extreme upper limit in the 32-hour material.

Helwig (1933) states that in irradiated *Circotettix* material he found fusion between the X and an autosomal fragment only twice. He explains this low frequency as due to the fact that the X is unpaired and relatively short. Since he does not state the frequency of autosomal fusions, it is not possible to say if this simple arithmetical explanation is sufficient. In the present work no undoubted cases of fusions between the X and an autosome have occurred (a possible, but doubtful case of such a translocation is illustrated in fig. 15).

In conclusion, one can say that the difference in reaction to irradiation between the X-chromosome and the autosomes is far greater in *Locusta* than in *Drosophila.*

## 10—THE TIME OF DIVISION OF THE CHROMOSOMES IN THE MITOTIC CYCLE

It is generally agreed that the chromosomes divide long before anaphase —that is to say that in prophase they consist of two chromatids lying side by side with a visible split between them. The only part of the chromosome which remains undivided up till prometaphase is the spindle attachment. One of the main controversies of modern cytology has been as to how long before anaphase the split becomes visible for the first time—*i.e.*, at what stage in the mitotic cycle do the chromosomes divide ? Two main view-points have been put forward: (1) that they divide during the resting stage, when their degree of dispersal is such that they cannot be studied, (2) that they divide at some time during the previous mitosis, either (2*a*) at metaphase or (2*b*) at prophase.

In the main the evidence which has been brought forward in support of these views has been direct observational evidence. Many workers have claimed to have seen a longitudinal split down the middle of anaphase chromosomes, or even an extra split (in addition to the obvious one) at prophase. Others (Darlington and co-workers) have denied the accuracy of these observations, being themselves unable to confirm them.

It should be pointed out here that the replacement of a single chromatid by two homologous ones lying side by side must necessarily involve two distinct processes, (1) the "copying" of the molecules in the original chromatid, one by one, and (2) the separation of these molecules into two chromatids which probably results from the surface charge on the whole structure becoming so great that instability results under the particular ionic conditions of the nuclear environment at the time (as suggested by Wrinch, 1934). The first is the essential and fundamental process of chromatid reproduction, the second may very well take place at different parts of the mitotic cycle in different organisms (thus explaining the discrepancies between the various workers).

Mather and Stone (1934) have attempted to solve this question of the time of splitting of the chromosomes by irradiation experiments. They obtained only " chromosome breaks " (*i.e.*, breaks of both chromatids at the same level) in the division following irradiation. They assumed (probably quite correctly) that the majority of the cells they studied must have been in the resting stage at the time of irradiation and explained their results by suggesting that splitting took place after irradiation, *i.e.*, during the resting stage. The work of Huskins and Hunter (1935) in which numerous single chromatid breaks were found seemed to be in contradiction to the argument of Mather and Stone. Huskins and Hunter also invoke direct observations to support their view that chromosomes split in the prophase of the division before that at which the chromatids so formed will separate. They conclude that the chromosomes are " longitudinally double at all stages, except just prior to anaphase-separation, when they are 4-partite."

The present work has, so far from solving the problem, shown that it is even more complex than has been hitherto suspected. Both " chromosome " and " chromatid " breaks were encountered and their presence at different times after irradiation noted:—

12, 17, 24, 28 hours—only chromosome breaks.

32 hours—chromosome and chromatid breaks.

51 hours and later—only chromosome breaks.

This suggests several possibilities, (1) that breakage can take place in both chromatids at the same level *after splitting* (this possibility, rejected by Mather and Stone on theoretical grounds, is suggested by the results with 12–28 hour material), (2) that there exists a stage in the cycle, about 32 hours before metaphase, and which we may assume to lie towards the end of the resting stage, when chromatid breaks and reciprocal chromatid fusions (§ 6) take place, for some reason, with great facility.

As far as direct observational evidence is concerned, no trace of a split down the anaphase chromosomes was encountered in the present work and no appearances were seen which were incompatible with the view that chromosomes split during the resting stage.

It is a pleasure to place on record my gratitude to Dr. B. P. Uvarov, of the Imperial Institute of Entomology, who supplied the animals used in this work. To Mr. F. Melville, Radiologist in the Anatomy Department of this College, I am indebted for the irradiation of the animals. To Professor J. B. S. Haldane, F.R.S., I am grateful for criticism and advice.

## 11—SUMMARY

X-rays produce both lethal and non-lethal effects in the spermatogonia of *Locusta migratoria*. Cells which are killed go into pycnosis. Those which are not killed show different kinds of chromosome abnormalities. The commonest of these is chromosome fragmentation.

Fragmentation of the X-chromosome is much rarer than autosomal breakage. The reason for this is not clear. No undoubted translocations between the X and autosomes were observed.

Ring-chromosomes and chromosomes with two spindle attachments result from fusion.

A new type of chromosome abnormality which leads to chromatid-tetraploidy with only a diploid number of spindle attachments is described. It is believed that this results from inhibition of the division of the spindle attachments following on irradiation.

The whole of the work strongly supports the view that the spindle attachment is a constant cell-organ and that new spindle attachments only arise from pre-existing ones. The independence of the spindle attachment from the rest of the chromosome in regard to time of division is emphasized by the new abnormality of mitosis described above.

Evidence is presented that the spindle attachments in the Acridiidae are not terminal, as hitherto believed, but sub-terminal.

## REFERENCES

Alberti, W., and Politzer, G. (1924). 'Arch. Mikr. Anat.,' vol. **100**, p. 83.

Crew, F. A. E., and Koller, P. Ch. (1932). 'J. Genet.,' vol. **26**, p. 359.

Darlington, C. D. (1933). 'Cytologia,' vol. **4**, p. 229.

Helwig, E. R. (1933). 'J. Morph.,' vol. **55**, p. 262.

Huskins, C. L., and Hunter, A. S. W. (1935). 'Proc. Roy. Soc.,' B, vol. **117**, p. 22.

Janssens, F. A. (1924). 'La Cellule,' vol. **34**, p. 133.

Kaufmann, B. P. (1934). 'J. Morph.,' vol. **56**, p. 125.

Koller, P. Ch., and Darlington, C. D. (1934). 'J. Genet.,' vol. **29**, p. 159.

Langlet, O. (1927). 'Svensk. bot. Tidskr.,' vol. **21**, p. 397.

Lewitsky, G. A., and Araratian, G. A. (1931). 'Bull. appl. Bot. Leningrad.' vol. **27**. p. 265.

Ludford, R. J. (1932). ' 10th Sci. Rep. Imp. Cancer Res. Fund,' p. 125.
Manton, I. (1935). ' Proc. Roy. Soc.,' B, vol. **118**, p. 522.
Mather, K. (1934). ' Hereditas,' vol. **19**, p. 303.
Mather, K., and Stone, L. H. A. (1934). ' J. Genet.,' vol. **28**, p. 1.
Muller, H. J., and Altenburg, E. (1930). ' Genetics,' vol. **15**, p. 283.
Muller, H. J., Prokofieva, A., and Raffel, D. (1935). ' Nature,' vol. **135**, p. 253.
Navashin, M. (1930). ' Univ. Calif. Publ. Agr. Sci.,' vol. **6**, p. 95.
—— (1931). ' Amer. Nat.,' vol. **45**, p. 244.
Prokofieva, A. (1934). ' Cytologia,' vol. **5**, p. 498.
—— (1935). ' Z. Zellforsch.,' vol. **22**, p. 253.
Rao, T. Ramachandra (1934). ' Proc. Ind. Acad. Sci.,' vol. **1**, p. 19.
Stadler, L. J. (1931). ' Sci. Agr.,' vol. **11**, p. 552.
Stone, L. H. A. (1933). ' Ann. Bot.,' vol. **157**, p. 815.
Sturtevant, A. H. (1931). ' Publ. Carnegie Inst. Wash.,' vol. **421**, p. 61.
Trankowsky, D. A. (1930). ' Z. Zellforsch.,' vol. **10**, p. 736.
White, M. J. D. (1934, *a*). ' Cytologia,' vol. **5**, p. 135.
—— (1934, *b*). ' J. Genet.,' vol. **29**, p. 203.
—— (1935). ' Naturwiss.,' vol. **24**, p. 390.
Winiwarter, H. de (1931). ' Arch. Biol.,' vol. **42**, p. 201.
Wrinch, D. M. (1934). ' Nature,' vol. **134**, p. 978.

545 . 21

# The Principle of Formaldehyde, Alcohol, and Acetone Titrations

## An Addendum

By GEORGE MAXWELL RICHARDSON

(From the Bland-Sutton Institute, Middlesex Hospital, London, W.1)

(*Communicated by C. R. Harington, F.R.S.—Received October* 15, 1935)

In a recent paper (Richardson, 1934, *a*), the extensive literature concerning the titration of amino-acids in presence of formaldehyde, alcohol, and acetone was examined. The intention was to outline the principles which underlie these titrations, and thence to deduce the experimental conditions which would be suited to particular analytical ends. In developing the argument, however, views were criticized which were thought to have been accepted by certain observers in describing their special techniques. It has since been pointed out that such criticism is liable to misunderstanding, and it is hoped by this note to remove any confusion unintentionally introduced into a matter already sufficiently involved.

The argument in the paper referred to was based throughout on the zwitterionic theory. Whilst this theory cannot be proved to be completely descriptive of the intermediate processes of titration, it nevertheless affords a basis for a simple description of the mechanism of titration which is in close accordance with the information at present available.

For purely analytical purposes, however (for example, estimation of all amino groups in a mixture), the only essential consideration is the stoichiometric correlation between the number of characteristic groups in the substance titrated and the amount of acid or base used between the initial- and end-points. In the titration of substances or mixtures containing several acidic or basic groups it is evidently necessary that statements of analytical results should clearly define what the initial- and end-points are. Some of the previous criticisms were occasioned by misunderstanding on this point.

Two common titration procedures, which differ in the initial point but resemble each other in the end-point, are in general use. The one, (*a*), is suitable for use in determining the amino-nitrogen content of biological fluids of unknown nitrogen composition—preferably without protein (Henriques and Sørensen, 1909; Richardson, 1934, *b*); the other, (*b*), is used to determine the changed content of amino or carboxyl groups in a solution in which these groups are appearing in known condition (as in proteolytic hydrolysates, Sørensen and Katschioni-Walther (1928)). The distinction between the procedure is described elsewhere (van Slyke and Kirk, 1933; Richardson, 1934, *a*). Unfortunately, certain authors, accustomed to the use of one or other of these procedures, have omitted to define the precise nature of the methods which they have employed. Thus Grassmann and Heyde's microtitration (1929) might easily be regarded as a titration by procedure (*a*), an interpretation accepted by Richardson (1934, *b*); yet the titration was presumably intended for use by procedure (*b*) with a technique adapted to minimize precipitation errors. Similarly, the supposition (Richardson, 1934 *a*) that the " Linderstrøm-Lang titration " would embrace an acidimetric acetone titration for carboxyl groups by procedure (*a*), was mistaken. Linderstrøm-Lang's intention to exclude procedure (*a*) from his method (1928, p. 1) was believed to refer to a determination of " excess of amino or carboxyl groups " by aqueous titration to about $p_H$ 6. Actually, the Linderstrøm-Lang titration was intended to be restricted to procedure (*b*), and was correctly designated " a method of determining amino-nitrogen."

## REFERENCES

Grassmann and Heyde (1929). 'Z. physiol. Chem.,' vol. **183**, p. 32.

Henriques and Sørensen (1909). 'Z. physiol. Chem.,' vol. **63**, p. 27.

Linderstrøm-Lang (1928). 'Z. physiol. Chem.,' vol. **173**, p. 32; 'Comptes rend. Lab. Carlsberg,' vol. **17**, No. 4.

Richardson (1934, *a*). 'Proc. Roy. Soc.,' B, vol. **115**, p. 121.

—— (1934, *b*). *Ibid.*, vol. **115**, p. 142.

Sørensen and Katschioni-Walther (1928). 'Z. physiol. Chem.,' vol. **174**, p. 251.

van Slyke and Kirk (1933). 'J. Biol. Chem.,' vol. **102**, p. 651.

547 . 64/68 : 535 . 343 . 3—3

*Abstract*

# The Ultra-Violet Absorption Spectra of some Complex Aromatic Hydrocarbons—I

By W. V. Maynebord, D.Sc., F.Inst.P., and E. M. F. Roe, B.Sc.

(International Cancer Research Foundation Scholar), Physical Section of the Radiological Department, and Research Institute, The Cancer Hospital (Free), London

The ultra-violet absorption spectra of ethyl alcohol solutions of a number of aromatic hydrocarbons, some of which are of biological importance, have been investigated. The compounds examined include benzene, naphthalene, anthracene, 1 : 2-benzanthracene, 1 : 2 : 5 : 6-dibenzanthracene, phenanthrene, and their derivatives, as well as chrysene, picene, pyrene and its derivatives.

Details are given of the experimental methods and some discussion of possible errors. The spectra of chemically related compounds are compared and general resemblances between them are pointed out.

In general no adequate theoretical discussion of the absorption spectra of these compounds is possible, but the effects of the addition of various groups have been noticed. Preliminary references are made to changes observed in the absorption spectra on cooling the solutions to approximately −183° C.

Some theoretical considerations of the subject are mentioned.

(*The full paper is published in* ' *Proc. Roy. Soc.,*' *A, vol.* 152, *p.* 299 (1935).)

547 . 64/68 : 535 . 343 .3—3

*Abstract*

# The Ultra-Violet Absorption Spectra of some Complex Aromatic Hydrocarbons—I

By W. V. Mayneord, D.Sc., F.Inst.P., and E. M. F. Roe, B.Sc.

(International Cancer Research Foundation Scholar), Physical Section of the Radiological Department, and Research Institute, The Cancer Hospital (Free), London

The ultra-violet absorption spectra of ethyl alcohol solutions of a number of aromatic hydrocarbons, some of which are of biological importance, have been investigated. The compounds examined include benzene, naphthalene, anthracene, 1 : 2-benzanthracene, 1 : 2 : 5 : 6-dibenzanthracene, phenanthrene, and their derivatives, as well as chrysene, picene, pyrene and its derivatives.

Details are given of the experimental methods and some discussion of possible errors. The spectra of chemically related compounds are compared and general resemblances between them are pointed out.

In general no adequate theoretical discussion of the absorption spectra of these compounds is possible, but the effects of the addition of various groups have been noticed. Preliminary references are made to changes observed in the absorption spectra on cooling the solutions to approximately −183° C.

Some theoretical considerations of the subject are mentioned.

(*The full paper is published in* ' *Proc. Roy. Soc.,*' *A, vol.* 152, *p.* 299 (1935).)

# Address of the President,
# Sir Frederick Gowland Hopkins, O.M.,
# at the Anniversary Meeting, November 30, 1935

It is my traditional duty to remind you of the losses the Society has suffered in the death of eighteen members. Four of these, Theobald Smith, Hugo de Vries, Friedrich Went, and H. F. Osborn, were distinguished Foreign Members. Among the fourteen Fellows are two who were active members of the Council, Dr. H. H. Thomas and Sir John McLennan.

By the death of Sir HORACE LAMB, the Society has lost a Fellow who for more than forty years was one of the most prominent and successful among the many workers in applied mathematics in this country. He was fortunate in his generation; Maxwell had shown the importance of the wave equation in electromagnetic theory; the work of Stokes, Rayleigh, and Thomson had aroused fresh interest in problems in heat and hydrodynamics, in all of which it was of importance. Lamb realized this and utilized his mathematical ability in the development of some of its many consequences. His papers on hydrodynamics and elasticity added to our knowledge in a marked degree. And in addition he was a great teacher. His text-book on " *Hydrodynamics*," the outcome of a course of lectures to undergraduates at Cambridge in 1875, is a model of what such a book should be, and the distinction of many of his pupils is clear evidence of the value of his work as a Professor at Manchester. To quote from a resolution of the Council and Senate, " He inspired all who knew him in the University with respect and esteem, and his many friends with warm affection."

THEOBALD SMITH was born at Albany, New York, on July 31, 1859. In 1884, he went to the Bureau of Animal Industry, U.S. Department of Agriculture as Assistant, and was made Director of the Pathological Laboratory in 1885. In 1895, at the age of 35, he went to Harvard as Professor of Applied Zoology, his chair being changed to one of Comparative Pathology in 1896. He also became Director of the Antitoxin and Vaccine Laboratory and Pathologist to the State Board of Health of Massachusetts. In 1915, at the age of 55, he became Director of the Department of Animal Pathology of the Rockefeller Institute for Medical Research at Princeton, New Jersey, where he stayed 20 years, retiring in 1929 at the age of 70. In each of the three foregoing environments (Washington, Boston, and Princeton) he built up great institutions and carried out investigations of great scientific importance. He died in New York on December 10, 1934, in his 75th year.

A list of his scientific publications, covering a period of 51 years, contains 255 titles. He did fundamental work on pathogenic organisms, their variability, adaptibility, mutations, differentiation, biochemical reactions, and advanced our knowledge of culture media and technique. He discovered that fluid cultures of the hog cholera bacillus, when injected into pigeons after being killed by heat (58–60° C), rendered them immune to inoculation with living cultures which killed control birds; this proved of much importance, for killed cultures have since been used on a vast scale for immunizing man against plague, cholera, typhoid, and paratyphoid fevers. He was the first clearly to differentiate tubercle bacilli of human and bovine origin. The results of his work on the toxin of the diphtheria bacillus and on antitoxin production, on accurately balanced toxin-antitoxin mixtures producing immunity for years without immediate or remote ill-effects, the finding that an excess of toxin in a mixture induced a much higher degree of immunity, etc., proved of great value when applied to man. He was the first to observe serum anaphylaxis (referred to by Otto as the " Theobald Smith phenomenon ").

His work on Redwater (" Texas fever ") of cattle, in which he discovered the causative protozoal parasite (*Babesia*) and its mode of transmission by ticks (*Boophilus*), followed by the successful application of preventive measures, has been of incalculable economic importance in many parts of the world. His discovery of the protozoal parasite (*Histoplasma*), causing " Black-head ", a deadly disease of turkeys in the United States, also of economic importance, was, like Redwater, of great scientific interest.

Finally, he was the first to demonstrate the significance of colostrum (first milk) to the new-born calf and why calves deprived of first milk are prone to die. He found that the blood plasma of the calf is devoid at birth of antibodies against common organisms like the colon bacillus. The colostrum contains such protective substances in high concentration, and, when ingested by the new-born animal, they are absorbed very readily into its blood which is thus armed against bacterial invasion. He was elected Foreign Member of the Society in 1932 and received the Copley Medal in 1933.

By the death of Sir Alfred Ewing on January 17, we have lost a colleague of rare distinction in many walks of life. He was in turn Professor in three Universities, Director of Naval Education, Principal and Vice-Chancellor of the University of Edinburgh, and in each position did educational work of the highest value. An Engineering laboratory, he claimed, endeavours, as all University Laboratories, to contribute to the common stock of knowledge by furnishing opportunities and appliances for original research. Magnetic hysteresis, re-discovered and so named by him when in Japan—he had been forestalled, though he did not know it, by Warburg, and some other German writers—will ever be linked with his name, while the discovery of slip bands made in conjunction with Rosenhain has been of great importance to metallurgists. His own view was that these results were due to good fortune rather than to any special merit. If he had any particular faculty it was that, he said, of grasping at the skirts of happy chance when they presented themselves; "They seemed to present themselves oftener when I was a young man than they do to young men now, but I dare say happy chance, like other women, wears her skirts shorter now."

In addition to his eminence as an investigator and teacher, he was a great administrator. To our own council, to the councils of other societies, and of the Department of Scientific and Industrial Research, he gave valued help; the National Physical Laboratory owes much to his continued interest in its progress. As a chairman he was most efficient. One who knew him well has written that "to work under Ewing was no sinecure; under a genial manner he had an infinity of driving power." Nor must a reference be omitted to his work for the nation in Room 40 during the war, when he deciphered, with the aid at first of ladies, "personal and family friends whose intelligence I knew and whose discretion I could trust," wireless messages picked up from enemy sources, an achievement characterized by Lord Fisher as one of the crowning glories of the Admiralty work during the war. The Society and the nation owe much to Sir Alfred Ewing.

Dr. FREDERICK AUGUSTUS DIXEY died in his eightieth year on January 16, as the result of injuries inflicted by a motor car in Park Lane. His earliest researches, on anatomical and medical subjects, were conducted at University College, London, where he collaborated with his brother-in-law, Sir Edward Sharpey-Schafer. Returning to Oxford in 1883, a chief interest of his youth was revived and he began, in the Hope Department of the Oxford University Museum, the long series of important researches on Lepidoptera, especially the Pierines (or "White Butterflies") of the world, on which he became the leading authority. His investigations, first published in 1890 and continuing with unabated energy until his death, were devoted to the evolution of wing-markings; the differences between wet- and dry-season forms; mimicry; the epigamic aroma of male butterflies; and to the structure and development of the scent-producing scales and of the wings which bear them. He will be long remembered as one who gave constant and efficient help to diverse causes, and whose presence promoted the pleasure and success of social activities in the scientific community.

By the death, in March this year, of Professor JOHN JAMES RICKARD MACLEOD medical science lost a man who, by his work, had contributed to the prolongation of the lives, in comfort and happiness, of hundreds of thousands of sufferers, otherwise doomed to an early death, from *Diabetes mellitus.* In 1921 the isolation of insulin, the active principle of the pancreas which, in some way, controls the utilization of sugar in the body, by a team of workers in the Toronto laboratory of which Macleod was head, added to the medical armamentarium a unique and potent product. Macleod's main research interest throughout his academic life was the metabolism of carbohydrate in the animal organism. No one more fitted both by experience and temperament could have been found to interest himself in the project of Banting when he suggested a fresh attack on the age-long problem of the relation of the pancreas to diabetes. The whole story of the discovery, isolation, and purification of insulin in the relatively short period it took is an outstanding example of what may be attained by the activities of a research team ably directed and concentrated on a single end.

By the death of Sir EDWARD SHARPEY-SCHAFER the Society loses its second oldest member. Elected in 1878, he twice served on the Council, and received the Copley and Royal Medals. Inspired by the teaching of Sharpey, he made his name first as a histologist, and became assistant Professor, and then Jodrell Professor of Physiology, at University College, London, and there became famous for his researches and in particular for those on endocrinology. Discovering the remarkable action of an extract of supra-renal gland on the blood pressure, he and George Oliver opened up a new field of physiological research which had important clinical applications. He became widely known to the general public as the initiator of the Schafer system of artificial respiration, which has been almost universally adopted for purposes of resuscitation. Transferred to the Chair at Edinburgh, Schafer gave his best in furthering both the interests of that University and the science of Physiology, and inspired his assistants with enthusiasm and affection. These are now scattered over the world and fill chairs in American, Dominion, and even Chinese Universities. On his retirement at the age of 83, twenty-nine of these contributed researches to an honour volume of the '*Quarterly Journal of Experimental Physiology*' which Schafer himself had founded and edited for many years. In the preface to this volume it is well stated that " there is no need to speak of what he has done; his contributions to physiological science are stored in the libraries and utilized daily by research workers in furthering the boundaries of knowledge which he has himself done so much to advance." A man of the highest integrity of mind, Schafer showed great power of lucid exposition, both in his text-books and as an oral teacher. Few men lived so long a life of usefulness to their fellows.

Sir JOHN ROSE BRADFORD was a great teacher of clinical medicine and he attained the highest office among clinicians, that of the President of the Royal College of Physicians. To University College and its Hospital he gave life-long service and was justly beloved by all who had been his pupils or patients. But at the beginning Bradford had been a physiologist and, to the great advantage of medicine, he never lost that outlook. His own research work dealt chiefly with the nature and distribution of what are now known as the autonomic nerves and it had no direct reference to problems of disease; its scientific merit was recognized by his election to our Fellowship at the age of 31. Later he passed over to clinical medicine and then, except for some thoughtful studies of the effects on metabolism of removing portions of the kidney, his experimental work ceased. But he kept in the closest touch with the biological sciences and deliberately gave himself to every administrative plan by which their advances could be used to help the progress of medicine in its own clinical science.

As a Secretary of our Society from 1907 to 1915, he was keenly interested in the work of the committees on Tropical Medicine and especially on sleeping sickness. His unique position of authority alike in science and in practical medicine made it natural that his advice and guidance should be sought for in all the movements that from 1900 onwards so greatly transformed the opportunities for medical research in London. His fairness in judgment, his straightforwardness and sense of honour, and finally his resolution gave him great power in persuading others to the changes that he desired. Had it not been for Bradford's influence, medicine in our islands would have moved all too slowly in the rear of physiology and the other sciences that were opening fresh roads for her advance, and the Medical Research Council at its creation would have found fewer men in the country to do its work.

CHARLES FREDERICK CROSS, who passed away at the age of 80, was one of those gifted individuals who combined the scientific spirit with technical ability and withal possessed an artistic temperament. His contributions to the subject of cellulose were many and important and for many years he was the leading scientific investigator into its nature and properties. At the same time his technical work led to continuous progress in the cellulose-using industries, particularly the paper industry. Outstanding for all time is his discovery of viscose and the development from it of artificial silk, which in its effect on the world at large is probably the most fruitful scientific discovery in its practical application since those of Faraday. Cross lived to see the people around him largely clothed in rayon, though the structure of cellulose is still only partly elucidated. He was a pioneer also in the field of cellulose acetate. The many scientific writings of Cross, particularly his original and now classical book on cellulose, showed an unusual gift of imagination, his technical work was of the highest precision and his consulting practice enjoyed a world-wide renown.

HERBERT BRERETON BAKER was born at Livesey near Blackburn in 1862 and during a long and active life carried out research work which materially advanced our knowledge of inorganic chemistry, especially in connexion with the inhibition of reactions in the absence of moisture. He was one of the few schoolmasters who, like Sir William Tilden, was able to reach high distinction both in Scholastic and Academic life, for he was elected a Fellow in 1902 while still a science master at Dulwich College. He was very fortunate in his teachers, because in early life he came under the influence of Francis Jones of the Manchester Grammar School, and later, when he was appointed Lee's Reader at Christ Church, Oxford, he became associated with H. B. Dixon who imparted to him some of the enthusiasm for research with which he was so amply endowed. After the German gas attack in 1915 Baker was sent with Dr. J. S. Haldane to carry out an investigation on the spot. He devised temporary measures of protection both against chlorine and phosgene, and although his methods have long been replaced by more perfect protective agents they were undoubtedly instrumental in giving adequate protection at a time when it was most needed. He was created C.B.E. for his war services in 1917.

JAMES WALKER was the first British student to be attracted to the new school of physical chemistry under Wilhelm Ostwald at Leipzig in 1887, and for nearly half a century thereafter sustained, through his research work and text-books, the role of the leading advocate of the subject in this country. Several of the more significant chapters of the van 't Hoff-Arrhenius theory of solutions—for example, those on hydrolysis and amphoteric electrolytes—were largely based on Walker's original work. He was also a skilled organic chemist, and rendered valuable service in the manufacture of T.N.T. during the Great War. The new department of chemistry at King's Buildings, completed in 1924, constitutes a fitting memorial to his twenty years' occupancy of the Edinburgh Chair. He was elected a Fellow in 1900, and served as Vice-President of the Society in 1930–31. In 1926 he received the Davy Medal.

HERBERT HENRY THOMAS belonged to that class of geologist which, through the inevitable expansion of the science, is becoming rare; for he combined a wide and expert knowledge of field stratigraphy with specialization in more than one branch of his subject.

After leaving Cambridge in 1898, with high honours, he acted for a time as Assistant to Professor Sollas at Oxford. In 1901 he joined the Geological Survey and was assigned to the South Wales district where Strahan was in charge. There his work lay amongst the igneous and sedimentary rocks of Lower Palaeozoic age. In country that had known De la Beche, Murchison, Logan, Phillips, Ramsay, and others, he was able with his colleagues to adopt Lapworth's threefold classification of the rocks and apply intensive zonal methods to their subdivision. On completion of the survey in South Wales in 1909, Thomas was transferred to the Flintshire Coalfield until 1911, when he was appointed Petrographer to the Survey and Museum. During the War his specialist knowledge was invaluable in the solution of various urgent problems for Government Departments, and in furthering the preparation of memoirs on the Mineral Resources of Great Britain. Thereafter he took an important share in the work of the Scottish branch of the Survey on the Tertiary igneous rocks of the Mainland and the Western Isles, the results of which have brought about one of the most impressive advances in British Geology made in recent years. Besides being the author of many important papers on stratigraphy, petrology, and mineralogy, his interest in pre-History was intense, as evidenced by his determination of the source of the "bluestones" of Stonehenge. Through his knowledge of mineralogy he became a pioneer in the study of the heavy minerals of sediments, his first thesis on this subject being the essay that won him the Sedgwick Prize in 1904. He was ever ready with advice, help, and encouragement; and his lovable personality secured him a host of friends. He became a Fellow in 1927, and at the date of his untimely death was a member of Council.

HECTOR MUNRO MACDONALD, who died on May 16 last at the age of 70, was a Royal Medallist of the Society in 1916, President of the London Mathematical Society in 1916–18, an Honorary Fellow of Clare College, Cambridge, and an Honorary LL.D. of Glasgow. He occupied the Chair of Mathematics in Aberdeen University for thirty years, and was an influential member of the University Court and of the governing bodies of many scientific and educational institutions in the North of Scotland. To pure mathematicians he was known as an investigator in the Theory of Functions, particularly the Theory of Bessel Functions; but most of his work was in Mathematical Physics. His rigorous solution of the difficult problem of diffraction by a prism was one of the most fruitful achievements in Optics in this century; and his determination of the effect, at a distant point of the earth's surface, due to a Hertzian oscillator emitting waves of a definite frequency, was the first important step towards an understanding of the propagation of wireless waves round the earth. He was elected a Fellow in 1901 and served on the Council in 1908–10.

HUGO DE VRIES died on May 21 at the age of 87. His early work on vegetable physiology culminated in a series of investigations on the causes of turgor in plant cells, by which he showed that it is due to osmotic pressure determined by the numbers of molecules and not by the weight of the solute. He thus provided independent demonstrations of the kinetic theories of solution associated with van 't Hoff and Arrhenius. Later he turned his attention to the experimental study of inheritance in plants and became one of the founders of the then infant science of genetics. He perceived that the material basis of heredity lay in the nucleus of the cell and as a consequence of his study of a chance population of *Oenothera* naturalized among the sand dunes near Hilversum he introduced the conception of ' Mutations '—large heritable variations in the organism by which varieties and presumably species originated, instead of the smooth flow of change by the accumulation of insensible steps which Darwin had postulated. This conception has become a permanent part of genetic theory. To de Vries as to few men was given the distinction of laying two foundation-stones in different branches of science.

JULIUS BEREND COHEN was one of the pioneers of this country in the teaching of organic chemistry. In his earliest days as a teacher he introduced practical courses in the subject and subsequently through the medium of his books was able to direct the steps of many students in their training as organic chemists. In addition to his great work as a teacher he was an enthusiastic research worker and was happiest when working himself or instructing others in his laboratory. His chief investigations were in the field of substitution in aromatic compounds, in problems concerning optical activity, and in the relation of chemical structure to antiseptic and trypanocidal action. He was one of those who as early as the beginning of the present century realized the contribution that chemistry could make in the study of biological problems, and many of his research students were directed into the field of biochemistry at a time when there were few workers in the subject. In some of his leisure time he sought by direct investigations on atmospheric pollution to convince his fellows of the ravages wrought by smoke in our industrial areas, and his work on this subject has been of great value. He was elected a Fellow in 1911.

Professor FRIEDRICH AUGUST FERDINAND CHRISTIAN WENT (1863–1935) began his career as a pupil of Hugo de Vries; after graduation he worked under Strasburger at Bonn and van Tieghem in Paris. After spending five years in Java he was elected Professor of Botany at Utrecht, a post which he held until 1934. In 1930, when the International Botanical Congress met at Cambridge, Professor Went received an Honorary Doctorate; shortly afterwards he was chosen by his Dutch colleagues to be President of the next International Congress, which was held in Amsterdam this year (1935). He looked forward with the keenest pleasure to the presidential duties and, despite failing health, he continued to exercise a controlling influence until a few days before his death, which occurred about a month before the date of the Congress. He was in an exceptional degree internationally minded and took advantage of every opportunity of promoting friendly co-operation and good feeling among biologists throughout the world.

The earliest important work undertaken by Went was a study of the distribution of sugars throughout the life history of crops of sugar cane in Java (1898), made on behalf of the growers in the Dutch East Indies. Went collected an imposing mass of analytical data for all parts of the cane, and for all ages and stages of growth. These data demonstrated the striking specific differences of behaviour between cane-sugar and the hexose sugars as the tissues of the plant develop. Thus was provided the first picture of the developmental drift of the carbohydrate metabolism of the plant. From this work in Java Went returned to Holland to take up the professorship of Botany and to direct the physiological research work of the Botanical Institute at Utrecht. On retiring from his professorship in 1934 he wrote an account in English (‘*Biological Reviews*,’ 1935) of the scores of investigations carried through in his Institute on the influence of the so-called “growth-substances” upon growth-rates and growth-curvatures. It is in connexion with this fine organization of continuous work on the first discovered hormone in plants that Went's name will be most widely known. This scheme of work progressively took up the measurement of the production of this hormone, the path and conditions of its transport, and the determination of its precise effect upon the complex phenomenon of cell-growth. Thereby it was made clear how the plant by its slower grade of transport and reactivity lends itself to the elucidation of certain fundamental problems of general physiology.

HAROLD WARD DUDLEY, who died before he had completed his 48th year, had acquired a wide influence in scientific circles concerned with biochemistry by his already fine record of investigation in that field, and by the unselfish devotion of his time and his work to the interests of his colleagues. He was one of a series of pupils of the late J. B. Cohen, who have won distinction in British biochemistry. He became early associated with H. D. Dakin at the Herter laboratory in New York, where from 1912 to 1914 they were jointly responsible for a series of brilliant investigations, particularly on the enzyme glyoxalase, which they discovered, and on the racemization of proteins. Dudley served with distinction during the war in the special organization dealing with protection against chemical warfare. After the war, he joined the staff of the National Institute for Medical Research as chief biochemist in the Department directed by Sir Henry Dale. He was there responsible for an important series of investigations on pituitary hormones, insulin, spermine, histamine, acetylcholine, and ergometrine, a new and important alkaloid from ergot which he isolated early in the present year, and of which his complete description was published in our '*Proceedings*' on the day of his death. Dudley did great service to biochemistry as Secretary of the Biochemical Society, and as Co-Editor of the '*Biochemical Journal*' at different periods. His early death removes a man whose personal qualities and unselfish devotion to the interests of all concerned with his subject had endeared him to a wide circle of workers in biochemistry and physiology.

In Sir JOHN CUNNINGHAM McLENNAN we lose one who in a special degree linked the rapidly growing scientific activities of Canada with those of the Mother Country. He began at Toronto University, with which he remained associated until the end. In his youth he came to Cambridge and gained a training in original research under Sir J. J. Thomson. Returning to Toronto, he took a prominent part in the development of physical teaching and research there, displaying an activity which can only be described as prodigious. After his appointment as director of the laboratory (1904) and professor (1907), nearly all that was done was under his inspiration and in many cases his personal direction as well. During the war his glowing patriotism showed itself. He developed processes for separation of helium from natural gas, and surveyed the available sources in Canada with a view to its use in airships and pilot balloons. Later, coming to England he developed methods of great practical value in the anti-submarine campaign. After the war he returned to Toronto, and did what was perhaps his best work. A cryogenic laboratory was set up, with arrangements for the liquefaction of hydrogen and helium, and he added notably to our knowledge of supraconductivity, showing that it is not in evidence when the current alternates rapidly. Further, he solved the long-standing mystery of the green line of the auroral spectrum, showing that it is due to oxygen and establishing the nature of the spectroscopic energy levels concerned. For this work he was awarded a Royal Medal in 1927. He received the K.B.E. in the present year (1935). His university recognized his services by appointing him Emeritus Professor and by giving the physical laboratory his name. His last years were spent in this country.

HENRY FAIRFIELD OSBORN will be widely regretted in this country, where he was well known, as well as in America. He has a special tie with London in that he attended a course on comparative anatomy given by Huxley fifty-six years ago. The American Museum of Natural History early recognized Osborn's value and under his curatorship the department of vertebrate paleontology became what it is to-day. His enthusiasm, professional skill, and adroit use of opportunity enabled him both in expeditionary work and in research to perform an almost incredible amount of successful work, well exemplified in his beautiful memoirs on Equidae and Titanotheres, while his numerous published papers deal with almost every branch of zoology and paleontology. He received the Darwin Medal in 1918 and was elected a Foreign Member in 1926.

This year's Report of Council reproduces the loyal Address of the Society to its Patron the King on the occasion of His Majesty's Jubilee, and also the gracious acknowledgment it received.

The Report as a whole makes it clear that the normal activities of the Society have proceeded during the year on satisfactory lines.

The Society remains fortunate in being entrusted with successive benefactions for the furtherance of research. A gift of £10,000 has been recently received at the instance of Professor P. E. Newberry and Mrs. Newberry from the trustees of the late William Johnston, shipowner of Liverpool, for the foundation of a fellowship or studentship devoted to research on the problems of Health, and, as its Report states, Council have been informed that the Society appears in the will of Mr. J. J. Jaffe as a possible beneficiary. The executors are at present seeking the authorization of the Courts before dealing with the estate. It is a striking fact that the Society's present expenditure in support of research is nearly tenfold that of twenty-five years ago.

Noteworthy also is the circumstance that in the last ten years the Society's publications have more than doubled in bulk; this is true of the '*Philosophical Transactions*' as well as of the '*Proceedings*.'

Under the wise guidance of the Treasurer, the system of accounting as applied to our funds as a whole has been reorganized and simplified. The change of method has already proved satisfactory and it will add to the convenience of all who wish to appraise the position of the Society's finances.

The problems associated with Dr. Kapitza's position, which has aroused interest and provoked comments in many countries besides our own, have now been solved on lines which the Royal Society, from its own standpoint, should, I think, regard as satisfactory. All Fellows of the Society will take pleasure in learning of the complete success which has attended the efforts of those responsible for the establishment of the memorial to Sir Charles Parsons.

The last few years have brought forth much discussion concerning the social responsibilities of science. One aspect of that theme has indeed become so familiar of late that I should hesitate to refer to it on such an occasion as this but for a particular circumstance. As most here will remember, the British Association recently listened to successive presidential addresses containing references to the misuse of the gifts of science. To discuss such matters is certainly proper to the defined functions of the Association, and, influenced chiefly by the circumstance just mentioned, and perhaps to some degree also by pressure from the lay press, which traditionally concerns itself in its own particular way with the business of the Association, its Council thought it well two years ago to invite various sections to devote part of their time to such discussion. At Aberdeen last year the problems involved certainly received some attention. Unfavourable comments have been made, however, because this year at Norwich it seems to have been already forgotten or neglected.

'*The Times*' itself in an article dealing with the Norwich meeting expressed regret for the neglect in question, and the writer ventured to say, apropos, that the Association revealed "a real hiatus" in its activities "in not studying science itself by scientific methods." This remark when first read seems to be an impressive one, at once an accusation and a suggestion for a line of action; but an endeavour to understand precisely what it means reveals, I think, considerable difficulties. Many, and not journalists alone, have called upon devotees of science to play some special part in helping to correct the social dislocations which their work is held to have caused, but I know of no concrete suggestions as to how, if as specialists, and not merely as good citizens, they are to set about it.

I will venture to quote very briefly from the presidential addresses which helped to bring this question so much to the front.

There came first certain striking remarks in the brilliant oration delivered by General Smuts at the centenary meeting of the Association in London. You will doubtless remember that he was dealing on broadest lines with the scientific world picture of to-day, but at one point he was led to remind his audience that " a serious lag has already developed between our rapid scientific advance and our stationary ethical development," a lag, he said, " which has already found expression in the greatest tragedy of history." In these words he provided the text for much subsequent discussion. He then went on to urge that Science itself must help to close this dangerous gap in our advance which threatens the disruption of our civilization and the decay of our species. " Its final and perhaps most difficult task," he concluded, " may be found just here." General Smuts, however, though in these words he emphasized the social responsibilities of science, spoke, as I shall immediately remind you, in a spirit of complete optimism concerning its ability to bear them. His immediate successor, Sir Alfred Ewing, perhaps because he was feeling the burden of the years, for his life of fine service was unhappily nearing its end when he delivered his address at York, spoke in a spirit less hopeful. He again emphasized the view that man is unprepared for the great bounty which science had provided for him, and expressed, you will remember, his own consequent disillusion when watching to-day the sweeping pageant of discovery and invention, in which as a young man he had taken unbounded delight. He expressed no hope that science itself could provide the remedy, but rather a failure to see from whence it was to come. We cannot fail to feel the pathos of such disillusionment in one whose death after a life of great accomplishment we mourn this year. We may believe that it was but a mood which, could he have lived to see the future growing brighter, might well have changed.

It was my privilege to follow these two distinguished men in the Chair of the Association, and I could not wholly forgo reference to the problems they had raised. I found, however, that from a humbler standpoint I could do little more than suggest that the inadvertent sins of science should be viewed in correct perspective, and to claim that biological science at any rate had done little to aid man's destructive impulsives but provided teaching that, were he willing to learn, might do much to correct these as well as other faults in his complex nature. This faith certainly remains with me.

Owing to what would seem to be a confusion in thought, the supposed social harms created by science have been vaguely attributed in some not unprejudiced quarters to its own unethical character. The conclusion reached by General Smuts, as we learnt from his address, is greatly different from this. He holds that among the values gradually recognized by the developing human mind, science ranks with religion and art, and that while to-day religion, art, and science are separate values, they may not always remain so. Indeed he thinks—so he told us—that one of the greatest tasks before the human race will be to link up science with ethical values. He admitted, it is true, that in speaking thus he was going beyond the scope of science as at present understood, but he expressed the belief that the conception of science itself is bound to be affected by its eventual integration with the other great values. I make no apology for recalling his words, for I think they should remain in our minds.

It is perhaps not quite easy to follow closely his thought concerning the future extension of the scope of science, but his own assurance that its teaching can be linked up with ethical values is a corrective for some of the accusations from which science is apt to suffer.

Indeed, possible future developments apart, the gap between what science provides for humanity and what are usually spoken of as values in religion, literature, and art, has been and is less wide than some assume. History itself shows that it is wrong to deny ethical influence, even if it be indirect, to the scientific spirit. While, for instance, the earlier stages of the Renaissance enriched men's minds by restoring to them the literary and philosophical heritage of antiquity, we all know that not until later, when the awakening scientific spirit demanded a courageous inquiry into the actual facts of Nature did human thought begin its release from the shackles of Authority and traditional dogma. This release was surely ethical in its effects. In later times throughout the years of the Royal Society's existence the growth of intelligent interest in the material universe was slowly preparing the ground for that last great step in the progress of intellectual freedom which came in the middle years of the last century. The influence of Darwin and Lyell did more, of course, than establish a new outlook for science itself. It re-orientated all thought. It caused heart-searchings where there had been mere complacency in the acceptance of tradition, and its impacts subtly encouraged the growth of that intellectual honesty for which Huxley and others then so nobly fought. The history of those days is familiar enough, but we sometimes forget the debt we owe to them.

It was the influence of science which, more than any other influence, established the idea of Progress, replacing as it did a static by an evolving universe, and incidentally a faith that man himself has an inherent capacity for advance. There are not a few to-day, including one very able and very articulate theologian, who profess to disbelieve in progress; but for the majority it is now a faith ingrained if sometimes only subconsciously held. Evolution we know may not at each step tend upwards, but we have reason to believe that in the long run it does, and that in spite of occasional disillusions we may believe in the upward tendencies of mankind. By this faith hope is fed, human efforts towards betterment are encouraged, and ethical values emerge. The depressing belief that Man and the social fabric he has contrived for himself, while so imperfect, are yet incapable of ultimate betterment, was the one excuse for that particular form of professed otherworldliness which from time to time has been an essential part of narrow religious ideals, but which was surely evil in its almost contemptuous indifference to social wrongs and to the urgent problems of this world.

With regard to values of the kind claimed in particular by art and literature, it is a prejudiced assumption that science must remain incapable of contributing to these. It is indeed right to insist, as Sir William Bragg has recently insisted in the course of a public lecture, that our perception of beauty need not be limited to that which appeals to our ordinary senses. The extension of the senses which science provides adds new beauties to the old without in the least degree supplanting them. There can be very few among true devotees of science who fail to feel this intensely. Sir Richard Gregory, indeed, in an eloquent Address on the " Interpretation of Science," urged the poets themselves to qualify for the emotional interpretation of these new beauties. He reminded his audience that at the beginning of the last century Wordsworth expressed his own assurance that when the right moment should come, the poet would find in the revelations of science a proper object for his Art, and went on to show by quotations that since Wordsworth's day many of our greater poets have had moments at least when the emotional value of those revelations has inspired them. General Smuts's faith that if to-day religion, art, and science are still separate values they may not always remain so, has a basis which is surely not unreal.

I will return for a moment to the circumstance from which I started and the criticisms it has called forth. You will have realized that the tendency of late has been not so much to reiterate the theme of science's gifts to the powers of destruction, but rather, with the supposed default of the British Association in illustration, to urge in a more general sense that scientific workers have not shown sufficient interest in the social implications of their work, or in the public responsibilities of their calling. We have only to look closely into this view as usually expressed to realize that, after all, its basis is vague, and, did it involve any charge against the individual worker, unreal.

It is after all not surprising, though the attempt was fully justified, that in the final issue members of the Association failed to find grounds for any very serious discussion of the subject. The individual investigator must realize that, *qua* scientist at least, he has little opportunity for effective action. Commonly he concludes that he will be most useful, even to society, by continuing his chosen work in its proper environment.

It is, I think, impossible not to sympathize with this view. The special endowments acquired by the scientific investigator are not those of a politician or of a missionary. Needless to say, it remains his duty to give his skilled services to the public whenever they are legitimately required and invited, and in so giving them he may do much to promote the interests of Science itself.

Although one may well believe that the recent extensive public discussion of the less happy social repercussions of science has had its uses, it has now fulfilled any purpose it may have had, and one may hope that on present lines at least it will now cease.

What seems to be really desirable is some method of closing the gap between the mind and outlook of the publicist and those of the trained scientist. It is a just claim that in a civilization so largely based on science as that of to-day, the scientist should have more influence on policy than he has hitherto been allowed. Not long ago the gap in question was wide; it is now, I think, lessening. Occasionally at least, modern statesmen do seek scientific guidance, and, I think, know better than they did how rightly to obtain it. But it is time, perhaps, that the building of a bridge should begin on the scientific side of the gap. This is a task for organized scientific effort and the pooling of knowledge. As John Stuart Mill urged, knowledge before it can assist public action must somewhere be concentrated.

Thirty years ago Sir William Huggins devoted the whole of a presidential address to a discussion of the advisory relations of the Royal Society to the State, pointing out how important these relations had been throughout the history of the Society. It must be recognized that during quite recent years its advice and assistance have been less sought by Government departments, or sought upon matters of relatively small importance. It is perhaps not difficult to understand why this should be the case. Other bodies, State-supported and qualified for advisory functions, have arisen; notably the three Research Councils under the Privy Council. The main business of these is to control nationally endowed research, but together they are highly qualified to give advice over a wide ground of technical problems, and they or their officials are in close touch with Government Departments. Moreover, the appointment of *ad hoc* Government Committees to deal with problems essentially scientific has lately become a very common policy.

It must be admitted that in these connexions the status of the Royal Society has been so far recognized that under the original Charters of the three Research Councils each election to their personnel must have the approval of your President *ex officio*; doubtless on the understanding that in any position of difficulty he would consult the Society through its Council. It is again because he represents the Society that the President sits on some of the Government Committees that I have mentioned. Naturally, many others among its Fellows are found on such committees. I do not indeed think that the Society should resent, or even regret that, as a body, it is less consulted than formerly on merely technical and highly specialized questions; but on broad general questions which bring science into contact with the State its constitution and prestige give it the right to be so consulted. I personally believe that such questions exist to-day, and with the growing influence of science on social developments they may well arise with greater urgency in the future. Should not the Society, with its unique authority, while always prepared to advise, be also perpared on appropriate occasions to approach the Government on its own initiative, and even at times make its own appeal to the public conscience?

There is a branch of scientific inquiry which can claim to yield knowledge of unqualified benefit to humanity, and one in which investigators, though inspired by the extreme scientific interest of the problems involved, have, I feel, always had the public importance of their solution in view, and have themselves done their best to encourage the practical applications of the knowledge they have won. I refer to the study of the nutritional needs of the body.

It must be confessed that up to the end of the last century this country had contributed too little to this side of knowledge, but British workers have since fully made up for that neglect.

The last two decades have seen a quite remarkable activity in research upon nutritional problems, and it is now even increasing. Publications describing original work upon various aspects of these come from almost every country where science is pursued, and have amounted to many hundreds during the course of each single year. A valuable journal, '*Nutrition Abstracts and Reviews*,' devoted, it is true, to animal as well as human nutrition, published in its first volume, issued in 1932, summaries of over 3000 papers and in its last completed volume over 4000.

In this country much research on nutrition has been generously endowed, organized, and encouraged by our Medical Research Council, and it has received financial support from various other sources. We have begun to follow the example of the United States in founding Chairs in Dietetics, and emphasis is being placed upon the subject in physiological and biochemical teaching at the Universities, while at centres where domestic science is taught, such as that at King's College, London, adequate knowledge is being supplied to students who afterwards in various capacities are imparting its essentials to the public. The excellent work of scores of infant welfare centres has educational value for mothers of the poorer classes. The British Medical Association has made it clear that the importance of nutrition is recognized by the profession, and clinical literature displays an increasing consciousness of the influence of malnutrition in the causation of ill-health. The Ministry of Health has now constituted a fully representative Committee on Nutrition, with advisory functions, and we may believe from many indications that the Government has awakened to the importance of the problems it involves.

Behind all these and other practical activities has been a somewhat sudden realization of the importance of right nutrition on the part of the educated community at large, and a consequent awakening of the public conscience and public concern with regard to it. There are but few to-day who doubt that it is the duty of the State to see that in this country no man or woman and, especially for the sake of the future, no child shall suffer inadequate nourishment unless for reasons beyond public control. It is much to be hoped that national interests of such importance will not be further exploited in party politics.

A desirable happening at the present moment is the international approach at Geneva for full discussion of the nutritional problems of the world; for, viewed broadly, the problems are undoubtedly international. As you doubtless know, on a demand from the representatives of twelve nations including Great Britain and Australia (which has played a leading part in the movement), the Health Organizations of the League have set machinery in motion for securing full and intimate discussion. Policies concerned respectively with the production, transport, distribution, and consumption of foods will all, we may hope, be discussed. They seem to be the very proper business of the League, and if discussion goes deep enough and is frank enough, it may well do no small service to the interests of peace itself.

Many are concerned just now to know the truth concerning the degree and extent of malnutrition as it exists among the less fortunate sections of the community at the present time. They have seen certain desirable steps taken to mitigate whatever may exist, as, for instance, the diversion of surplus milk to the schools; but they yet ask how far is an extension of such measures an urgent need of the moment. That underfeeding and ill-feeding exist is sure, but to measure their extent is, for reasons to which I will allude, a task of some difficulty.

We have only to read the successive annual reports from numerous Medical Officers of Health to believe that, in the case of children at least, nutrition, inadequate for one reason or another, exists in various districts to an extent which is far indeed from being negligible. Nevertheless, we have to realize that the numerical data supplied from different centres are based upon varying standards, and the opinions expressed depend to no small degree on the temperament and perhaps on the acuity of individual officers. The difficulty before all concerned is that there is no clear definition of the term "malnutrition," and no satisfactory objective methods for measuring its degrees. Sir George Newman, the late Chief Medical Officer to the Ministry of Health, always felt justified in taking an optimistic view concerning the available food supply of children in this country, and he was wont to insist upon the circumstance that though under-nourishment may be due in part to an insufficient quantity of food, it depends also on other factors. "The principal cause," he once wrote, "is a body unable to assimilate the food supplied to it." In certain cases this must, of course, be true, but we are entitled to ask whether in many of such cases the disability of the body when observed has not followed upon malnutrition at an earlier period. What we know to-day justifies this view. In any case the essential question is whether food adequate in quantity and quality is within the reach of all. In the Annual Report for 1934 issued by the present Chief Medical Officer of the Ministry, it is made clear in a section devoted to the effect of unemployment on national health and dealing with evidence based upon a conjoint investigation by officers of the Ministry and the Board of Education into conditions in certain depressed areas in Durham, that the statistics of actual diseases there do not indicate unfavourable effects of present economic conditions. We may conclude from this, and take comfort from the fact, that malnutrition even in such areas has not been at its severest, but it may be highly deleterious in its ultimate effects when not such as to affect the current statistics of disease. Moreover, full normal nutrition was only claimed for some 70 to 80 per cent of the children, varying between these figures in different areas, and though, as I have said, criteria for establishing the existence of subnormal nutrition are not satisfactory, it is difficult to be content with such figures. The Ministry's investigators admit further that in the areas studied the condition of adolescent youths, especially those aged 14 and 15 years, was found unsatisfactory. These are years when good nutrition is quite specially desirable. As a measure of the state of health of younger adults at the present time, the results of medical examinations for recruiting are not without importance. The War Office Report on the Health of the Army for 1933 shows that in that year nearly 38 per cent of the prospective recruits were rejected as unfit. Although, as the Report points out, many of these rejections were on account of disabilities which unfitted them for military service in particular, and therefore did not reflect on their general health, yet a glance at the stated reasons for rejection shows that a large proportion of the men suffered from defects which might well be due to faulty nutrition in childhood or adolescence, and a considerable proportion in which it almost certainly contributed to their unfitness.

I have read most of the available literature bearing on this important matter, and have tried to appraise the value of the evidence it offers. I am left with a strong impression that the situation calls for further and immediate action.

The current interest in the subject of the national food supply and in right feeding is doubtless largely due to the awakening of the public conscience to this and other kindred social responsibilities; but it has also been stimulated, I think, by the nature of the results which scientific studies during the last twenty years have revealed. They have shown, as you know, that our nutritional needs are so much more numerous, subtle, and specific than was earlier thought, and they have shown how extraordinarily potent and how entirely indispensable material may be, even though consumed in infinitesimal amounts. We know now that a fault in quality may be as deleterious as a failure in quantity. This fact the general public is now rapidly assimilating though not always to its profit. Indeed, a certain vocal section of the public is (as it has always been) so perverse in its views concerning food that it is almost necessary to remind it that after all quantity still counts. We cannot live on vitamins alone! A more intelligent section of the public seems, I notice, to assume with impatience that so much scientific talk about food may lead to individuals being dragooned with respect to what they shall or shall not eat. A self-constituted Committee whose propaganda against malnutrition I respect for its wisdom and fairness has appreciated this attitude, and in a recent memorandum made the following wise statement: " certain diet habits, must be classed as cultural, and of personal and psychological necessity, and to upset these in the service of protein or vitamin content is to provoke a natural reaction against " the advice that science can legitimately give. It is, of course, unthinkable that there should be any such dragooning. The intelligent public will learn to apply the newer knowledge of nutrition without discomfort to itself.

The same memorandum says—again wisely—" to a working class housewife with restricted purchasing power, education in food values alone can only add one further problem to an already insoluble series of anxieties and worries." This of course is true, and so long as we see that foods of the right variety are all within her purchasing power, elementary instruction in terms of the foods themselves and not in technical food values will set the housewife on the right path. I am tempted to add that what the English housewife in the poorer classes needs most to be taught is the art of simple but good cooking ! It is not beneath the dignity of nutritional science or of administrative policy to take note of the circumstance that in this country more than in perhaps any other is good food ruined, and its nutritional value impaired, by unintelligent treatment in the home.

One last consideration before I release you. Is the time yet ripe for the initiation of a comprehensive National Food Policy; one that will endeavour to adjust production, in a qualitative as well as a quantitative sense, to right consumption, and at the same time organize all the details of distribution on national lines ?

I note that those responsible for the contents of that very impressive book entitled " *The Next Five Years*," which has appeared under the aegis of many influential names, hold that no administrative action less comprehensive than a national policy of the kind in question could deal adequately with existing problems. They suggest that it should now be taken in hand. Others have urged that, apart from the almost prohibitive magnitude of effort the policy would require, it is one which should await the arrival of more knowledge. The latter suggestion has, I think, but little point. There is doubtless much more scientific knowledge to be gained about nutrition and food production, but we know enough to guide administration on to the right lines.

More to be thought of perhaps is the fact that if we take a long range view any policy concerned with food production must ultimately, if it is to be ideal, become part of a world policy. You may have noticed that at Geneva Mr. Bruce, speaking of such a world policy, said that its aim would be to " marry agriculture to health." Such a marriage, if ever properly consummated, would greatly profit the world. The interests of both partners must be equally guarded, however, in the marriage contract. In this country one may venture to say the first mentioned is at present receiving preferential treatment.

My last official duty as your President will be the pleasant one of distributing the medals of the year. Before proceeding to this, however, I would like to express my deep gratitude to the Society for having entrusted me with an office so highly privileged and so much to be coveted. If I have fulfilled its duties with any success it has been due to the continuous support and help I have received from my fellow officers to whose unremitting care for the interests of the Society I wish to testify. To all members of Council who during my term of office have shared in the heavy responsibilities which ultimately fall to that body my gratitude is also due. Finally, I wish to thank the Assistant Secretary, Mr. Winckworth, and all the members of the office staff for their loyal and efficient services.

My retirement is associated with that of Sir Henry Dale, whose ten years of fine service as Secretary will remain outstanding in the records of the Society. I am sure he joins with me in wishing success to those whom for the moment I must call our probable successors.

## *Awards of Medals*, 1935

Professor CHARLES THOMSON REES WILSON is awarded the COPLEY MEDAL. He has contributed fundamentally to the progress of modern physics by his work on the use of clouds in advancing our knowledge of atoms and their properties. How wide the field of his work has been can be seen from the following summary of his researches. He studied the phenomena of condensation of vapour to form drops by the use of expansion apparatus—which he designed. He discovered the existence of "rainlike" and "cloudlike" condensation of water vapour and measured the corresponding supersaturations and the corresponding critical expansions. He also measured the supersaturations requisite to produce condensation of water vapour in ions of both signs produced in various ways. He devised and constructed various types of electrometers and with them and with his cloud apparatus demonstrated the so-called natural ionization of air and other gases. Later he made valuable contributions to our knowledge of atmospheric electricity. With a new form of expansion apparatus, he photographed the tracks of ionizing particles and radiations traversing gases. He next developed methods of measurements which he applied to the study of thunderstorm electricity. In this work the main phenomena relating to the electric field changes produced by lightning discharges were observed for the first time, studied quantitatively, and the principles of their interpretation outlined. He further extended the use of the cloud-chamber method. Stereoscopic methods of photographing ionization tracks were applied successfully to the investigation of the properties of photo-electrons and Compton recoil electrons including their ranges. In recent years he has continued to make numerous and important contributions to the study of atmospheric electricity and, by introducing important improvements and modifications into his cloud-chambers, he has greatly extended their use.

A ROYAL MEDAL is awarded to Professor CHARLES GALTON DARWIN, F.R.S., for his researches in mathematical physics, especially in quantum-mechanics, optics, and statistical mechanics.

His papers on the reflexion of X-rays are now regarded as the fundamental researches on the subject. In his first paper he obtained the formula which is now accepted as generally applicable. There emerged, however, a contradiction: the theory predicted an intensity far less than the experimental. This caused him to discuss the subject more deeply; he traced the discrepancy to its source, and was thus led to investigate the imperfection of crystals, a subject which he was the first to treat effectively.

In a series of papers on statistical mechanics, written in collaboration with R. H. Fowler, he published a new mathematical technique by which the relation of thermodynamics to statistics was placed on a satisfactory basis, and the way was opened for extensive subsequent developments.

Perhaps the most original and influential of Darwin's papers have been those concerned with quantum-mechanics. The quantum theory of the motion of an electron in an electromagnetic field, as it had been developed before 1925, gave results not in accordance with experiment. To obviate this, in 1925 the assumption was introduced that the electron has a mechanical angular momentum and a magnetic moment; but this conception did not admit of the application of wave-theory, since the waves corresponding directly to such an electron would be in space of six dimensions. It was Darwin who in February, 1927, put forward the idea which led ultimately to the solution of these difficulties; he assumed that, just as there are two independent polarized components in a wave of light so there are two independent components in the wave of an electron. He then constructed a pair of equations to represent the fine structure of the hydrogen spectrum, obtaining all the levels correctly except the *s*-levels, and showed how the equations could be expressed in vectorial form, so that, as he expressed it, the electron was a vector wave. This paper was the first important contribution to the modern wave-mechanics of the electron.

Among the quantum papers is also numbered the celebrated memoir on *Free motion in wave mechanics*, in which he took up the general question of aperiodic phenomena and dealt with it in masterly fashion, the principles of description and interpretation which are now universally accepted being formulated and applied to the free motion of electrons and atoms in various types of field. Coming as it did at a critical time, this paper had a great influence.

The reader of Darwin's papers is impressed by his original and powerful mind, his immediate penetration to the heart of a problem, and his combination of deep intuition in physics with mastery of analytical methods.

A ROYAL MEDAL is awarded to Dr. ALFRED HARKER as the greatest British petrologist since that subject became a science; his original contributions have had a profound influence throughout the world. In 1886 he published a paper on Slaty Cleavage, which added considerably to the better understanding of the nature and laws of cleavage phenomena. His next important contribution was the Sedgwick Prize Essay of 1889 on the Bala Volcanic Series of Carnarvonshire. In collaboration with his colleague, J. E. Marr, he published a notable work on the Shap Granite and associated rocks. It is probably true to say that Harker's greatest achievement is the geological mapping of the island of Skye from 1895 to 1901, the results of which were published as a Memoir of the Geological Survey in 1904. This monumental work has had a very wide influence upon the science of petrology: in this Memoir and in the subsequent Memoir on the Smaller Islands of the Inner Hebrides, Harker set a high standard of work both in the field and in the laboratory; he settled the long-standing controversy between Geikie and Judd, and his results have been confirmed by subsequent workers. His book on the "*Natural History of Igneous Rocks*," published in 1909, has been described as "a work which has had a greater effect than any geological publication in the last fifty years." He has also made contributions of great value to the subject of differentiation of rock magmas, etc. His latest book on "*Metamorphism*," published in 1932, is the best contribution to the subject written in any language.

Professor ARTHUR HARDEN is awarded the DAVY MEDAL in recognition of his distinguished work in biochemistry and especially of his fundamental discoveries in the chemistry of alcoholic fermentation.

His researches form a landmark in the history of the organic chemistry of biological processes.

In 1904, in collaboration with his colleague W. J. Young, he showed that the alcoholic ferment of active cell-free yeast juice, the preparation of which had been achieved a few years earlier by E. and H. Buchner, could be separated into two components, a thermolabile non-dialysable enzyme complex and a thermostable dialysable co-enzyme, both of these being essential for the fermentation of sugar. They observed that the fermentation of sugars by this yeast juice was profoundly affected by an addition of inorganic phosphate and followed up this discovery by a detailed and systematic investigation of the carbon balance, from which they showed that for each hexose molecule broken down to carbon dioxide and alcohol another molecule became esterified with phosphoric acid. From the fermentation products, hexosediphosphoric and hexosemonophosphoric esters were subsequently isolated by Harden and his colleagues. These discoveries have been the foundation upon which much of our present knowledge of the intermediate changes in the enzymic breakdown of carbohydrate has been established. In muscle and other glycolytic systems, utilization of carbohydrate is effected by processes closely similar to those occurring in yeast and involving the intervention of phosphate and formation of phosphoric esters. Harden's monograph on alcoholic fermentation is a masterly discussion of this problem of yeast metabolism.

Harden has made important contributions to our knowledge of chemical reactions effected by bacterial enzymes, especially those concerned in carbohydrate metabolism. He has also taken an active part in the development of vitamin research in this country, while as editor of the '*Biochemical Journal*' since 1911 he has influenced deeply the development of this branch of chemistry.

A HUGHES MEDAL is awarded to Dr. CLINTON JOSEPH DAVISSON for his discovery that electrons are diffracted like waves of light. Like many other discoveries, that one disclosed itself gradually; but its origin can certainly be traced to some experiments on the scattering of electrons by nickel made by Davisson and Kunsmann in 1921. Davisson recognized at the time that the observed effects did not harmonize with the existing theories of matter and electrons. Owing to the polycrystalline nature of the metals investigated, the correct interpretation to be put upon the results was not at all clear. Davisson fully recognized the importance of this discrepancy, and this he and his assistants proceeded to investigate with great pertinacity and skill.

Towards the end of 1926 Davisson and Germer succeeded in making similar experiments with a single metallic crystal surface so that the results were simple enough to be interpreted easily. It was then at once seen that the electrons were diffracted like waves of light of the wave-length required by the theory which had in the meanwhile been put forward by M. de Broglie towards the end of 1924. The first announcement of Davisson and Germer's results was published in '*Nature*,' April 16, 1927. This publication was the first clear experimental proof of the wave character of the electron.

Davisson and his associates, principally Germer, have confirmed, amplified, and extended these results in numerous later publications. Davisson has also made important contributions to thermionics.

577 . 158 . 43

# Uricase, Amino Acid Oxidase, and Xanthine Oxidase

By D. KEILIN, F.R.S., and E. F. HARTREE, Ph.D.

(From the Molteno Institute, University of Cambridge)

(*Received October* 21, 1935)

## I—INTRODUCTION

The object of this paper is the comparative study of three oxidizing enzymes—uricase, amino acid oxidase, and xanthine oxidase. We shall describe first the main properties of uricase and amino acid oxidase, laying special stress on characters which have not been sufficiently investigated by previous workers. This will include the study of the effect of various factors on the activity of these enzymes, the reaction between these enzymes and their substrates, the activation of the substrate molecules, their reaction with the molecular oxygen, and the reduction of the latter to hydrogen peroxide. We shall then examine briefly the main characters of xanthine oxidase, and this will enable us to compare the properties of these three enzymes and to determine some of the characters they have in common.

## II—URICASE OR URICO-OXIDASE

### 1—*Previous Work*

That uricase or urico-oxidase catalyses the oxidation of uric acid to allantoin has been known since the work of Schittenhelm (1905), Wiechowski and Wiener (1909), and others, but its main properties have been established only by Battelli and Stern (1909, 1912) in their important investigation on this subject. According to these authors, for the oxidation of a molecule of uric acid to allantoin one atom of oxygen and one molecule of water are taken up while one molecule of $CO_2$ is given off. The reaction consists, therefore, in oxidation, hydration, and decarboxylation, and the R.Q. of the reaction is usually equal to 2. It varies slightly, however, according to the age of the enzyme preparation. The relationship which these authors have established between the amount of uric acid disappearing, the oxygen taken up, and the $CO_2$ given off, has made possible the study of the reaction by the estimation of either oxygen or $CO_2$. The velocity of oxidation of uric acid was found to depend on the oxygen tension, being, for instance, at least twice as great in pure oxygen as in air. The main results obtained by Battelli and Stern have been recently confirmed by other workers, who have, however, paid special attention to the study of the kinetics of this reaction (Felix, Scheel, and Schuler, 1929; Schuler, 1932; Rô, 1931; Grynberg, 1931). The velocity of the reaction was measured by these authors in terms of the amount of uric acid oxidized, of oxygen absorbed, and of $CO_2$ liberated; and these reactions were studied at various hydrogen ion concentrations, at different tensions of oxygen, and at different concentrations of enzyme and substrate. The oxidation of uric acid catalysed by the enzyme was also compared with that obtained by permanganate and by hydrogen peroxide. One of the important conclusions which resulted from this work was that the enzyme does not catalyse directly the oxidation of uric acid to allantoin, but that the reaction takes place in two steps: (1) the catalytic oxidation and hydration of uric acid by uricase to an oxy-acetylene-diurein carboxylic acid, and (2) the decarboxylation of this unstable compound to allantoin, which is independent of the enzyme (Biltz and Schauder, 1923; Felix and his co-workers, 1929; Grynberg, 1931; and Schuler, 1932).

## 2—*Material and Methods*

Uricase can be easily prepared from fresh ox kidney or even better from pig's liver. The best method of preparation is similar to that which is used for zymine preparation. Fresh pig's liver, after being carefully cleansed from ligaments and fat, is minced by means of a Latapie mincer, the pulp stirred with six volumes of acetone for 5 minutes, and then filtered on a large Büchner funnel. The dried residue is mixed with two volumes of acetone which is carefully sucked off and the dry product is spread in a thin layer on filter paper and left overnight at room temperature. The next day the yellowish dry product is collected, ground in a mortar into a fine powder, and stored in a vacuum desiccator. This powder keeps its activity for several months.

The oxidation of uric acid was tested manometrically by measuring the oxygen uptake and the liberation of carbon dioxide in Barcroft differential manometers, shaken in the usual way in a bath at 39° C. In our experiments, the measured amount of the enzyme preparation, suspended in buffer solution, is put in the right-hand flasks of the manometer and made up to 3 cc with buffer solution. The uric acid or sodium urate is carefully weighed in small cups provided with platinum hooks which are suspended from the potash tubes of the flasks. The potash tubes (unless mentioned otherwise) receive 0·3 cc of 10% KOH and a roll of filter paper. The left-hand flasks receive only 3 cc of buffer solution, and 0·3 cc of KOH with a roll of filter paper in the central tube. After equilibration on both sides of the apparatus, the taps of the manometers are closed and the cups are dislodged, liberating their contents into the flasks. In a series of manometric experiments with this enzyme, we have first confirmed the main results obtained by Battelli and Stern and other workers, namely, that in the oxidation of one molecule of uric acid to allantoin one atom of oxygen is taken up and one molecule of $CO_2$ is given off, the R.Q. of the reaction being approximately 2.

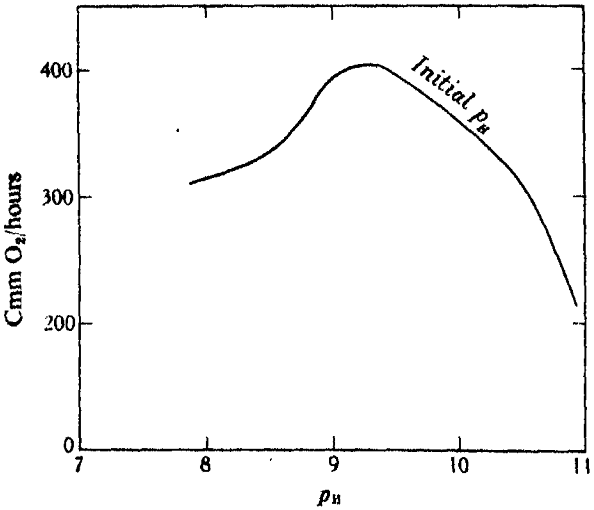


FIG. 1—Effect of $p_H$ on the initial rate of oxidation of uric acid, 5 mg, by uricase, 50 mg acetone powder, at 39° C.

### 3—*Effect of $p_H$*

In the study of the effect of $p_H$ on uricase, the reactions were carried out in phosphate and borate buffer solutions mixed with the enzyme preparation, the $p_H$ of the mixture being determined with the hydrogen electrode at the beginning and at the end of the experiment. It was noticed that at the end of the experiment the $p_H$ of the solution drops markedly, and, at about the end of the reaction, the $p_H$, according to the buffering capacity of the solution, drops by 0·7 to 1·6 $p_H$ units. For instance, a solution buffered at the beginning of the experiment to $p_H$ 9·2 shows at the end of the experiment $p_H$ 8·3. The optimal $p_H$ of activity of the enzyme prepared from pig's kidney lies at about 9·25 estimated by the initial rate of oxidation, fig. 1. We failed to confirm the existence of two distinct maxima at $p_H$ 8·9 and 9·9 found by Felix *et al.* (1929) from the estimation of the velocity of uric acid decomposition.

### 4—*Effect of Inhibitors*

*a—Effect of KCN and its Reversibility*—That uricase is inhibited by small concentrations of cyanide, was shown independently by Truszkowski (1930) and by Fosse and his co-workers (1929, 1930). Truszkowski believed, however, that this inhibitory effect is irreversible. In a series of experiments with various uricase preparations, we have found that $10^{-3}$ M KCN inhibits the enzyme activity by 94%, and that even $3 \times 10^{-5}$ M KCN inhibits it by 83%. However, contrary to Truszkowski's results, we have found that this inhibitory effect is perfectly reversible, even when strong concentrations of cyanide are used. That the inhibition is reversible is shown by the shape of the curves representing the velocities of oxidation of uric acid in presence of various initial concentrations of cyanide, fig. 2. Such curves are usually obtained when manometric measurements of oxygen uptake are carried out in flasks containing rolls of paper soaked with strong KOH, which gradually absorbs the HCN liberated from the solution. The reversibility of cyanide poisoning can

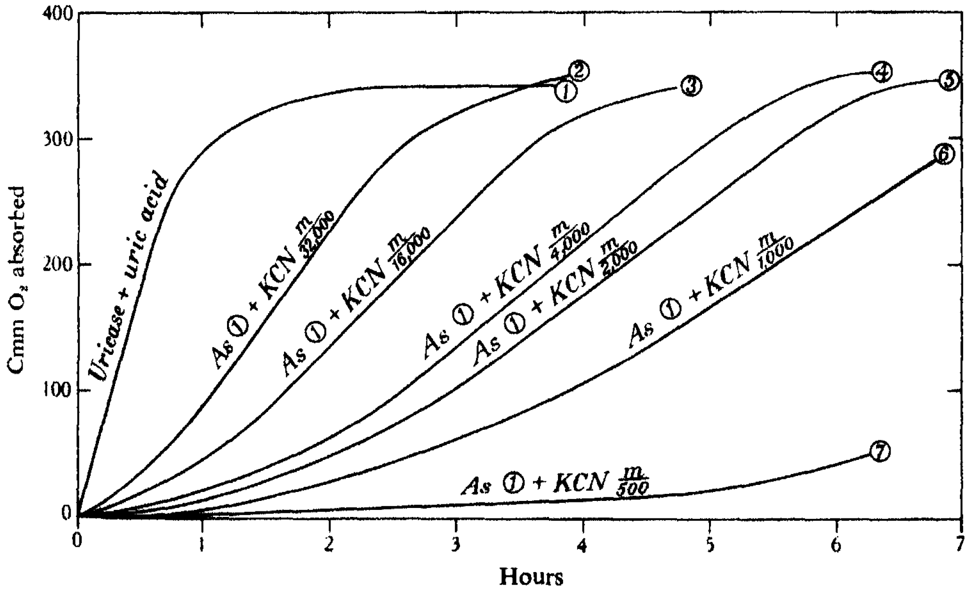


FIG. 2—Effect of various concentrations of KCN on the oxidation of uric acid by uricase showing the reversibility of inhibition due to the absorption of HCN by strong KOH.

also be demonstrated by another method, based on the ability of methaemoglobin to combine with cyanide giving cyanmethaemoglobin, the formation of which requires one molecule of cyanide per atom of iron of methaemoglobin. The experiments were carried out in four Barcroft manometers, the left flasks of which received 3 cc of buffer solution and 0·3 cc of KOH. The right flasks of all the manometers received the usual amount, 50 mg of enzyme preparation, 5 mg of uric acid, and KOH in the central tubes. The flasks of three manometers (2, 3, and 4) received $10^{-3}$ M cyanide and the side bulbs of two of them (3 and 4) received also

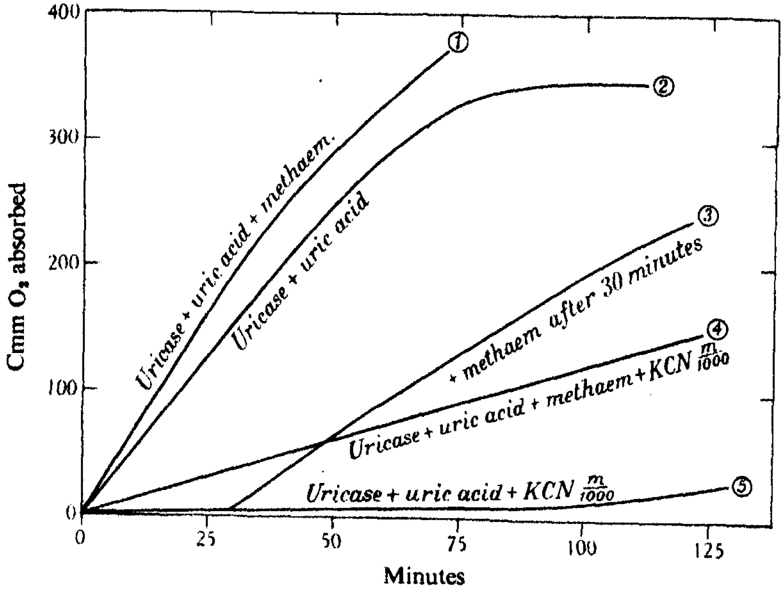


Fig. 3—Reversibility of KCN poisoning of uricase: Uricase, 50 mg; uric acid, 5 mg; methaemoglobin, 0·5 cc of 10%; KCN $10^{-3}$ M.

0·5 cc of 10% solution of freshly recrystallized horse methaemoglobin. The methaemoglobin from the side bulbs in manometer 3 was delivered into the flasks at the beginning of the experiment and in manometer 4 thirty minutes later; the readings of the manometers were taken every 10 minutes. The results of these experiments, fig. 3, show that cyanide produces very strong inhibition which is almost completely abolished if methaemoglobin is added 30 minutes after the beginning of the experiment. If, however, the methaemoglobin is added at the beginning of the experiment, the inhibitory effect of cyanide, although much reduced, is not completely abolished. This, at first sight, unexpected result can be easily explained. In manometer 4 during the first hour of experiment, a large amount of cyanide has already been absorbed from the solution by the KOH. The methaemoglobin added therefore removed only the last traces of inhibitor. In manometer 3, cyanide, not having been removed by KOH, combined with methaemoglobin, forming a fairly stable cyanmethaemoglobin compound. There is in this flask a definite partition of cyanide between the enzyme and methaemoglobin which is kept up during the whole period of the experiment. Cyanmethaemoglobin forms in this case a reserve which maintains a very small constant concentration of KCN. The reversibility of cyanide poisoning can be demonstrated not only with a suspension of acetone enzyme preparation but also with the clear alkaline extract of enzyme, completely free from solid particles.

*b—Effect of other Inhibitors*—Several other substances have been tried as inhibitors in the usual way and it was found that CO ($CO/O_2 = 8$), $Na_2S$ (M/250), NaF (M/10), and urethane (5%) had very small or no inhibitory effect. M/500 $NaN_3$ (sodium azide) at $p_H$ 8 inhibits the activity of the enzyme by 28% and at $p_H$ 6·8 by 40%. Hydroxylamine at the fairly high concentration of M/50 only inhibits the enzyme by 45%, and pyrophosphate, even at concentrations between M/50 and M/25, slightly increases the activity of the enzyme. Of other substances tried in concentrations of M/250, 5-nitro-4-chlor-2-aminophenol, and 1-amino-2-naphthol-6-sulphonic acid both produced about 45% inhibition of the enzyme, while 2-aminophenol-4-sulphonic acid and 8-oxyquinoline sulphonic acid inhibited the enzyme by only 10–14%. The addition of 0·2 mg copper or iron inhibited the enzyme activity by 45 and 82% respectively. This inhibition is abolished when the metals are added in an M/12 phosphate buffer containing M/30 pyrophosphate.

### 5—*Specificity of the Enzyme and Competitive Inhibitors*

One of the important properties of uricase is its very high specificity. Of all the known purine compounds, it oxidizes only uric acid. It does not promote the oxidation of other purines such as hypoxanthine or xanthine which are so easily oxidized by xanthine oxidase, nor the various derivatives of uric acid such as 4: 5-glycol-, 4: 5-dimethyluric acid, 1-, 3-, 7-, or 9-methyluric acids (Brüning *et al.*, 1928). We have tested 16 mono-, di-, or tri-methyl or ethyl derivatives of uric acid and found that none of them was catalytically oxidized by this enyzme.* We have found, on the other hand, that several of these derivatives produce a great inhibitory effect on the oxidation of uric acid by uricase, figs. 4 and 5. For instance, 5 mg of 1 : 3 : 7-trimethyl uric acid depresses by 68% the initial rate of oxidation of 5 mg uric acid by 100 mg of uricase preparation at $p_H$ 8·9. Other methyl derivatives under similar conditions inhibit the activity of uricase by 16–60% and the inhibition increases with the concentration of the methyl compound, fig. 5. The fact that the methyl compounds of uric acid, although not oxidizable by the enzyme, inhibit the oxidation of uric acid shows that these methyl compounds react

---

* The samples of these compounds we owe to the kindness of Professor Biltz, to whom we address here our thanks.

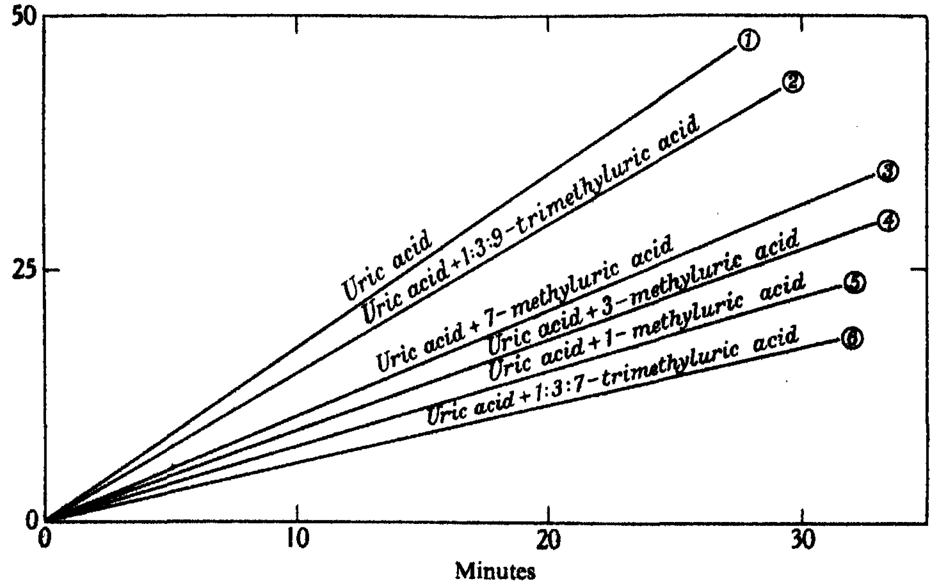


FIG. 4—Effect of substituted uric acids, 5 mg, as competitive inhibitors of uricase + 5 mg uric acid.

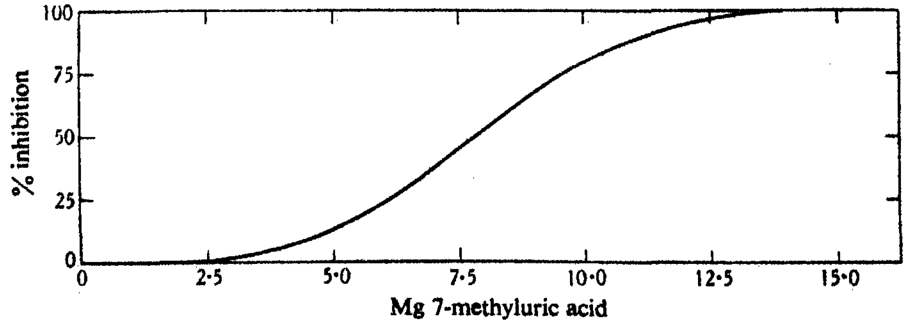


FIG. 5—The progressive inhibition by 7-methyluric acid of uricase + uric acid, 5 mg.

with the same active grouping of the enzyme molecule as uric acid itself. As a result of this reaction, uric acid becomes activated but, the methyl compounds not being activated, can act only as competitive inhibitors.

### 6—*Formation of* $H_2O_2$ *and Secondary Oxidation of* p-*phenylenediamine*

In several experiments on the oxidation of uric acid by uricase it was found that the total amount of oxygen taken up was somewhat higher than the amount required for the transformation of uric acid into allantoin, and that the R.Q. of the reaction was less than 2. It was obvious that although the uricase preparation alone had no oxygen uptake, it contained some substances which underwent oxidation in the presence of uric acid and that this oxidation was not accompanied by the formation of carbon dioxide. This excess of oxygen uptake was never larger than 20% of the theoretical uptake, but it was easily increased by the addition of various substances. The best results were obtained by the addition of *p*-phenylenediamine which appreciably increased the oxygen uptake of the system. This excess varied, however, from one preparation of uricase to another. While in some preparations the addition of phenylenediamine increased the theoretical uptake of oxygen by only 20%, in others it increased the uptake by at least 80%. If, however, to the system uricase + uric acid + *p*-phenylenediamine, which shows only a small excess of oxygen uptake, we add a small amount of peroxidase preparation, the oxygen uptake becomes twice that produced by the enzyme and uric acid alone. For instance:

| | Uptake of $O_2$ cmm |
|---|---|
| Uricase + uric acid (theoretical $O_2$ uptake = 330) ...... | 362 |
| Uricase + uric acid + *p*-phenylenediamine ............ | 424 |
| Uricase + uric acid + *p*-phenylenediamine + peroxidase | 757 |
| Uricase + *p*-phenylenediamine + peroxidase .......... | 40 |

These experiments, *see also* fig. 6, clearly show that *p*-phenylenediamine is oxidized by hydrogen peroxide formed during the oxidation of uric acid to allantoin. In other words, we are dealing here with a secondary or coupled oxidation similar to those described by Thurlow (1925) and Harrison and Thurlow (1926). According to these authors, $H_2O_2$ is formed during the oxidation of hypoxanthine to uric acid by xanthine oxidase. This peroxide in the presence of a peroxidase can bring about the secondary oxidation of nitrites (Thurlow) or *p*-phenylenediamine (Harrison), while in the presence of ferrous salts it can oxidize lactic acid to acetaldehyde or β-hydroxybutyric acid to acetoacetic acid. In our experiments, the mere addition of *p*-phenylenediamine produces at once a substantial increase in the oxygen uptake by the system. This increase is undoubtedly due to the peroxidatic activity of haematin compounds present in uricase preparations. On the other hand, the peroxidatic property of haematin compounds is not very marked and does not prevent some of the hydrogen peroxide formed in the primary reaction from undergoing decomposition by the catalase present in the preparation, or from being used in the oxidation of other molecules of uric acid activated by uricase. In the presence of a real peroxidase, which has a very great affinity for $H_2O_2$, all the peroxide formed during the primary reaction is directly used in the oxidation of *p*-phenylenediamine, with the result that the oxygen uptake becomes twice that produced in the primary oxidation reaction. The fact that the hydrogen peroxide is formed during the process of oxidation of uric acid by uricase suggests that the reaction proceeds according to the following equation:

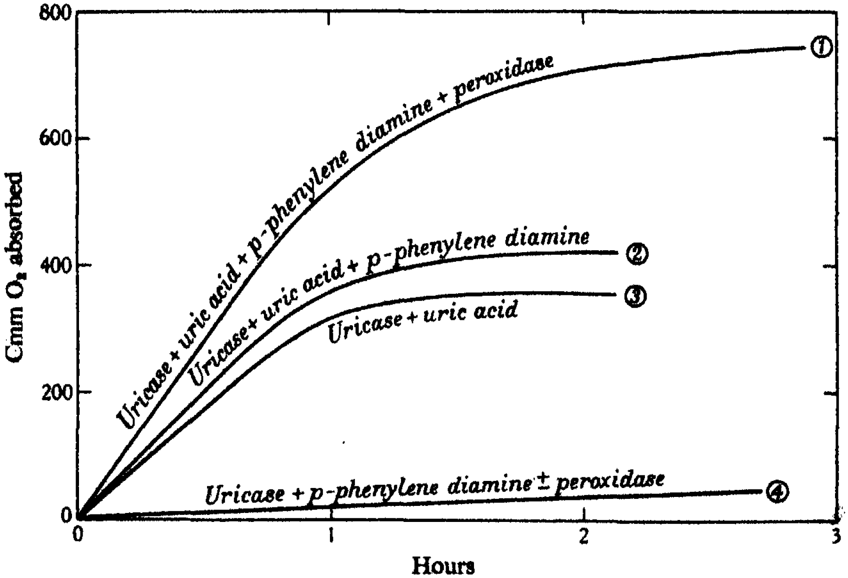


FIG. 6—Coupled oxidation of *p*-phenylenediamine by the uricase system.

$$\text{Uric acid} + 2H_2O + O_2 = \text{allantoin} + CO_2 + H_2O_2.$$

In this reaction it is possible that the first step consists in the formation of 4 : 5 glycol uric acid through the oxidation of the two carbon atoms joined by a double bond and a simultaneous rearrangement of the molecule into oxy-acetylene-diureine carboxylic acid (Biltz and Schauder, 1923), which undergoes decarboxylation giving rise to allantoin. Although Brüning *et al.* (1928) have failed to obtain allantoin from 4: 5-glycol uric acid fed to dogs or perfused through dog's liver, it is conceivable that this derivative, when formed under biological conditions, rapidly undergoes the changes mentioned above.

It is generally believed that an enzyme which is poisoned by KCN, and which, with the substrate, does not bring about the anaerobic reduction of methylene blue,* belongs to the category of aerobic oxidases (Przylęcki and Truszkowski, 1928) activating the oxygen which in oxidizing the substrate does not become reduced to hydrogen peroxide. This generalization does not, however, apply to uricase which, as we have seen, being strongly and reversibly inhibited by KCN, activates the substrate molecule, which reacts with molecular oxygen reducing it to hydrogen peroxide.

The secondary oxidation of *p*-phenylenediamine can be considered only as a very convenient method for detection of $H_2O_2$ formed in the primary oxidation reaction. Among other substances which have been tried as substrates for the coupled oxidation, the most interesting results have been obtained with alcohols. It was found that under certain conditions the hydrogen peroxide formed in the primary reactions can oxidize alcohols to their corresponding aldehydes or ketones. This reaction was found, however, to be more complicated than the oxidation of *p*-phenylenediamine and will be discussed in a separate paper.

## 7—*Secondary Oxidation of* p-*phenylenediamine and Indophenol Oxidase*

The fact that *p*-phenylendiamine and " Nadi " reagents can be oxidized by a peroxidase and the $H_2O_2$ formed in the primary oxidation reaction was used by Harrison (1929) as an argument against the necessity of postulating the existence of an independent indophenol oxidase. According to Harrison, the indophenol reaction of heart muscle preparation may be explained by the secondary or coupled oxidation of " Nadi " reagent by $H_2O_2$, formed during the oxidation of substances like glutathione or hypoxanthine. That this view is not correct is clearly shown by the following considerations: (1) in heart muscle preparation, which can be considered as an example of a typical indophenol oxidase, no peroxidase can be detected apart from cytochrome, haematin, and some derivatives of haemoglobin which give a peroxidase reaction only on addition of $H_2O_2$ and benzidine. (2) If we assume that $H_2O_2$ is constantly formed in the washed muscle preparation, the oxygen uptake of this preparation alone would be about half of that which is given by the preparation in the presence of *p*-phenylenediamine. We have found that in a powerful enzyme preparation the oxygen uptake is either nil or represents only about 0·5% of that which it shows in the presence of *p*-phenylenediamine. (3) The oxidase reaction of the preparation and its capacity of oxidizing cytochrome remain for a long time (several weeks) unchanged, even if the preparation is frequently aerated. During such a period, if the preparation contained substances undergoing primary oxidation, they would all be completely removed by oxidation. (4) The presence of $H_2O_2$ in oxidase preparation cannot be detected even on the addition of strong horse-radish or milk peroxidase together with benzidine. (5) The indophenol oxidase is extremely sensitive to drying, which rapidly destroys this enzyme, while this factor does not affect the enzymes promoting the formation of $H_2O_2$ such as xanthine oxidase, uricase, and amino acid oxidase. (6) Among the inhibitors such as KCN, $H_2S$, $NaN_3$, and CO, which inhibit the activity of the indophenol oxidase, CO has no effect on other enzymes including peroxidase, while of the three other poisons either one or the other only acts on each of the enzymes which may be responsible for the formation of $H_2O_2$. Although *p*-phenylenediamine and " Nadi " reagents can be used for the detection of $H_2O_2$ formed in the primary reaction of oxidation by the enzymes such as uricase, the positive indophenol reaction thus obtained has no bearing at all on the problem of the indophenol oxidase. This criticism may be applied to all similar deductions based on a single reaction such as oxidation of a suitable substrate without analysing the conditions under which the reaction can proceed or is inhibited. We must remember that the mere fact of an oxidation of a substrate whether it be *p*-phenylenediamine, alcohol, or glucose, obtained by different catalytic systems, does not imply the identity of these systems with those which produce analogous reactions in living cells. The identification can be made only after a careful analysis of the mechanism of the reactions and a study of the conditions under which it occurs or is inhibited *in vivo* and *in vitro*.*

---

* The experiments which we have carried out with the usual Thunberg vacuum tube method confirm the results of previous workers (Przylęcki and Truszkowski, 1928, and others), namely, that the uric acid in the presence of uricase and in the absence of oxygen does not reduce methylene blue. We have found, moreover, that even the dyes of a higher $r_H$ such as indophenols, do not react with this system.

---

* It may be mentioned here that an attempt was made recently by Remesow (1932) to identify indophenol oxidase with lipoid substances such as cholesterine because the emulsion of this substance in water or its solution in chloroform is supposed to oxidize a mixture of *p*-phenylenediamine and *p*-tolylenediamine. Remesow writes:

## III—AMINO ACID OXIDASE OR *d*-AMINO ACID DEAMINASE OF KREBS

It was shown by several workers that different organisms are capable of bringing about the oxidative deamination of the aminoacids. More recently, this oxidation has been demonstrated manometrically on slices of liver and kidney by Kisch (1931) and especially by Krebs (1933) who, moreover, succeeded in separating from tissues a cell-free enzyme capable of catalysing the aerobic deamination of different amino acids. To Krebs we owe also a careful quantitative study of the reaction promoted by slices or by the soluble enzyme and the study of the main properties of this enzyme. The following are the main results obtained by this author (1933, 1935):—(1) Both slices and extracts of some tissues such as kidney oxidize the amino acids aerobically to the corresponding keto acids. The ratio $O_2$: $NH_3$: keto acid being approximately equal to 1 : 2 : 2. According to Krebs, there are in cells of various organs, but especially in kidney, two enzyme systems promoting the deamination of amino acids. (1) A system which can be separated from cells and which is not inhibited by KCN, octyl alcohol, or drying. This system, named by Krebs *d*-amino acid deaminase, is capable of oxidizing only the non-natural optical isomers of the amino acids, specially the monocarboxy monoamino acids. (2) A system which cannot be separated from cells, which, like the respiration of cells, is strongly inhibited by KCN and by octyl alcohol and destroyed by drying. This system, called by Krebs l-amino acid deaminase, oxidizes the natural amino acids. According to Krebs (1935), it may be possible that these two systems have certain components in common and that the soluble enzyme of *d*-amino acid deaminase,* for instance, may be a component of the l-amino acid deaminase system.

" In diesem Sinne ist es nicht ausgeschlossen dass das gefundene Sterin-system dem Warburgschen Eisen-system sehr nähe steht und eine massgebende Rolle bei den biologischen oxydativen Prozessen spielt " or " die von Battelli entdeckten und beschriebenen ' Oxydone ' nichts anderes sind als das im Organismus katalytisch wirkende Cholesterin im kolloidalen oder molekularen Zustand." This supposition can, however, be easily dismissed as being based so far on crude observations of colour reactions without comparative estimations of oxygen uptake. Cholesterin preparations, moreover, do not appreciably oxidize the ordinary reagents such as *p*-phenylenediamine (and " Nadi ") and, what is more important, they do not oxidize the reduced cytochrome *c* which is so easily oxidized within the cell or by muscle preparation. In cholesterin experiments no tests have been made with any of the oxidase inhibitors such as KCN, $H_2S$, $NaN_3$, and CO, without which it is superfluous to discuss any parallelism between the behaviour of an indophenol oxidase and that of various artificial catalytic systems.

* This enzyme was previously described by Krebs under the name of amino-acid-oxido-deaminase. This term should not be confused, however, with a similar term introduced by Bach (1914) to designate a hypothetic enzyme component of tyrosinase.

More recently, it was shown by Bernheim and Bernheim (1934) that, in the presence of amino acid oxidase, all the simple amino acids reduce methylene blue, but that the rate of the reduction varies considerably with the amino acid and is independent of the corresponding rate of oxygen uptake. This fact was confirmed by Krebs, who found that the rate of anaerobic deamination of amino acid by methylene blue is very much lower than that of the aerobic deamination by oxygen.

In the course of our work we have confirmed the main results obtained by Krebs and other workers and have revealed also some other properties of this enzyme which have not been previously recorded.

### 2—*Material and Methods*

The enzyme is easily prepared from the kidneys of ox, pig, or sheep; the cortical layer of the kidney is carefully removed, minced with the Latapie mincer and mixed with 7 volumes of pure acetone. The mixture is stirred for about 5 minutes and filtered on a Büchner filter. The residue is mixed with two volumes of acetone which is completely sucked off. The almost dry residue is collected, spread in a thin layer on filter paper, and allowed to dry at room temperature. The dry acetone preparation is ground to a fine powder which will keep its activity for several weeks. For each series of experiments 1 gm of this powder is mixed with 40 cc of buffer solution, left standing for about 20 minutes, which ensures almost complete extraction of the enzyme, and cetrifuged. Of the clear solution 2 cc is used for each Barcroft flask and corresponds to 50 mg of dried acetone preparation. On standing, the activity of the extract gradually falls. It was found to lose about 35% of its activity during each of the first 2 days. The study of the activity of this enzyme, as with uricase, was carried out in Barcroft differential manometers.

### 3—*Effect of Substrate Concentration*

The effect of concentration of substrate on the rate of oxidation was determined only for *dl*-alanine. The results of these experiments, when plotted, give a rectangular hyperbola, fig. 7, such as has been obtained for many other enzymes. The substrate concentration, giving half the maximum velocity (or the Michaelis constant Km) obtained from this curve is approximately $5 \times 10^{-3}$ M.

### 4—*Effect of Inhibitors*

*a—Irreversible Effect of $H_2S$*—It has been shown by Krebs (1933, 1935) that the enzyme which promotes the oxidation of *d*-amino acids is not inhibited by cyanide. We have confirmed this observation and found that cyanide, if anything, slightly increases its activity. We have found, on the other hand, that the enzyme is strongly inhibited by very

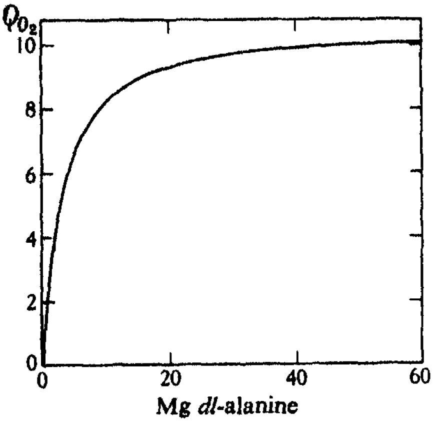


FIG. 7—Relationship between rate of oxidation by amino acid oxidase, 50 mg, and concentration of *dl*-alanine.

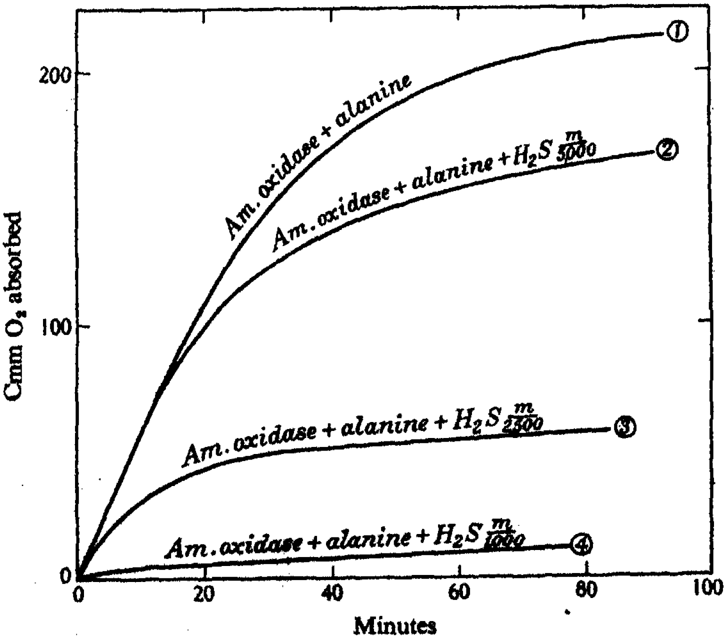


FIG. 8—Effect of $H_2S$ M/250 on amino acid oxidase 50 mg + *dl*-alanine, 5 mg.

small concentrations of $H_2S$, fig. 8. This inhibition, as shown in fig. 9, differs, however, markedly from the usual type of inhibition by specific poisons. The inhibition appears to develop gradually on incubation of the enzyme with $H_2S$, but what actually happens is that during this period oxygen is being rapidly absorbed and the $H_2S$ oxidized to completion. In subsequent experiments it was found that the quantity of oxygen absorbed in absence of substrate was very greatly in excess of that required for oxidation of the sulphide to sulphate. It thus appears that the enzyme is destroyed during the oxidation of the $H_2S$ as it remains completely inactive after the disappearance of the $H_2S$. The rapid initial oxygen uptake shown in curves 2 and 3 of fig. 9 is therefore due to simultaneous oxidation of $H_2S$ and alanine. Further evidence in favour of this view was obtained by incubating the enzyme with $H_2S$ for 1 hour in absence of $O_2$ at 39° C and $p_H$ 6·5. After removing all free $H_2S$ by evacuation, the enzyme activity was found to be unimpaired.

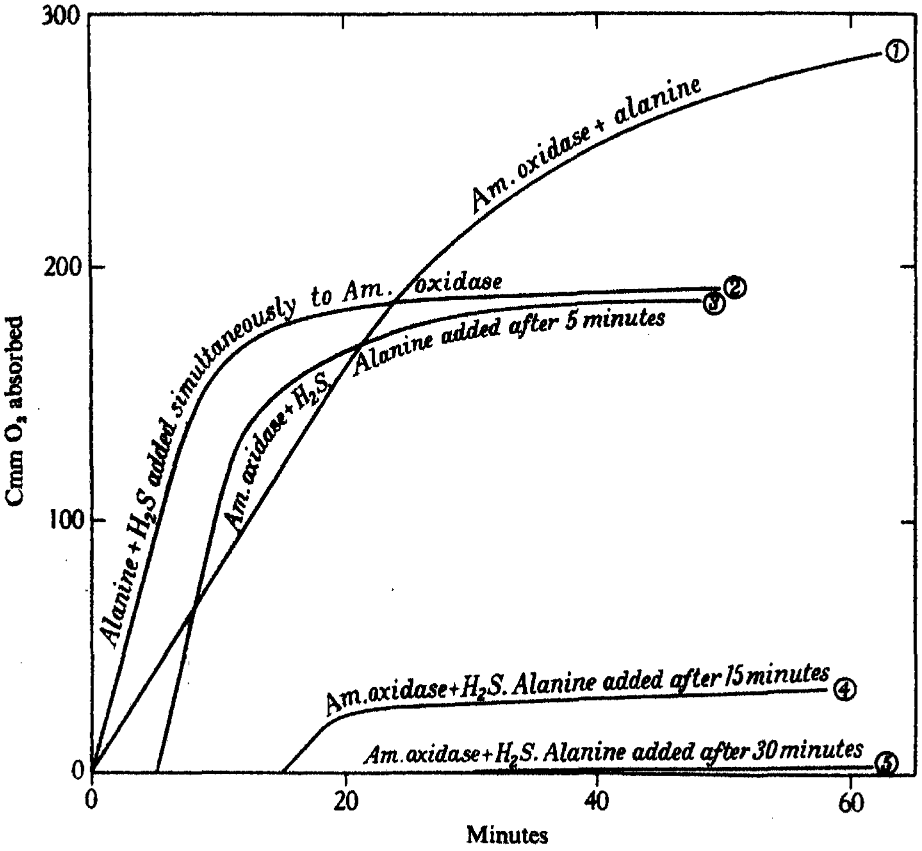


FIG. 9—Effect of incubation with $H_2S$, M/500, on amino acid oxidase.

*b—Effect of other Inhibitors*—The effect of other substances on this enzyme has been tried in the usual way by adding them to the enzyme before the substrate which was usually alanine. 0·57 M ethyl urethane completely abolishes the activity of the enzyme, 0·28 M of this narcotic inhibits it by 70%, 0·14 M by 24%, and 0·057 M by only 12%. As, on the other hand, octyl alcohol (Krebs, 1935) and 0·003 M phenyl urethane have no effect on the enzyme, while 0·84 M NaCl inhibits the enzyme irreversibly by 83%, the inhibition produced by ethyl urethane may be only of the nature of a salt effect. Pyrophosphate 0·02 M has no effect on the enzyme, αα-dipyridyl produces very slight inhibition (10%), sodium azide ($NaN_3$) at $p_H$ 7·8 produces if anything a slight activation, even in concentrations as high as 0·01 M. At $p_H$ 6·8, 0·002 M azide inhibits the enzyme by about 53%. Cysteine, on the contrary, has no effect on the initial rate of the oxygen uptake but increases the total oxygen uptake, which, as we shall see, is due to the secondary oxidation of this substance.

The amino acid oxidase is very sensitive to a great variety of other substances which can act neither as specific poisons nor as indifferent narcotics. We have seen previously that the enzyme can be easily extracted with water from the acetone preparation of kidney. The activity of the clear enzyme solution is strongly inhibited by the addition of the residue of the extracted powder (Krebs, 1935). This inhibitory effect is not due, however, to the presence of specific inhibitors in this powder, as it can be obtained by the addition of a great variety of extracts or solid particles obtained from other tissues. The enzyme is, for instance, inhibited by the addition of uricase preparation of liver, oxidase preparation from heart muscle, or catechol oxidase preparation from mushrooms. The strongest inhibition was obtained, however, with xanthine oxidase prepared from milk. The addition of 50 mg of xanthine oxidase (caseinogen preparation) freed from salts, to the amino acid oxidase (extracted from 50 mg of acetone powder) inhibits the initial rate of oxidation of alanine by 50 to 68%. Moreover, the inhibition rapidly increases and the oxidation of alanine is soon completely abolished, the final oxygen uptake reached representing not more than 20% of the theoretical uptake. This inhibitory effect is greatly reduced by boiling xanthine oxidase for only a few minutes, fig. 10. Other preparations of xanthine oxidase produce still greater inhibitory effect, for instance, the addition of a suspension of 80 mg of whey preparation, washed free from salts, inhibits the amino oxidase by 98%. That this inhibition by xanthine oxidase is not due to the specific action of this enzyme on amino acid oxidase can be easily demonstrated. It was shown that the property of xanthine oxidase to oxidize hypoxanthine to uric acid is completely abolished after 30 minutes' incubation of the enzyme with cyanide at

39° C.* On the other hand, such an inactive enzyme preparation still possesses the property of inhibiting the amino acid oxidase. Moreover, the inhibitory effect similar to that of xanthine oxidase is also shown by ordinary charcoal. 50 mg of Merck's medicinal charcoal added to the enzyme ($p_H$ 7) inhibits its activity by at least 92%, while the same amount of gelatinous calcium phosphate or of Kieselguhr at the same $p_H$ have no effect on the enzyme. It is possible that the inhibition of amino acid oxidase by extracts of tissue or various enzyme preparations is mainly due to the introduction of colloidal surfaces having the properties of adsorbing the amino acid oxidase, its substrate, or both. It may be mentioned also that the incubation of the enzyme with 2 mg of crude pancreatin preparation rapidly abolished the activity of the oxidase, while incubation with boiled pancreatin has no inhibitory effect, fig. 11.

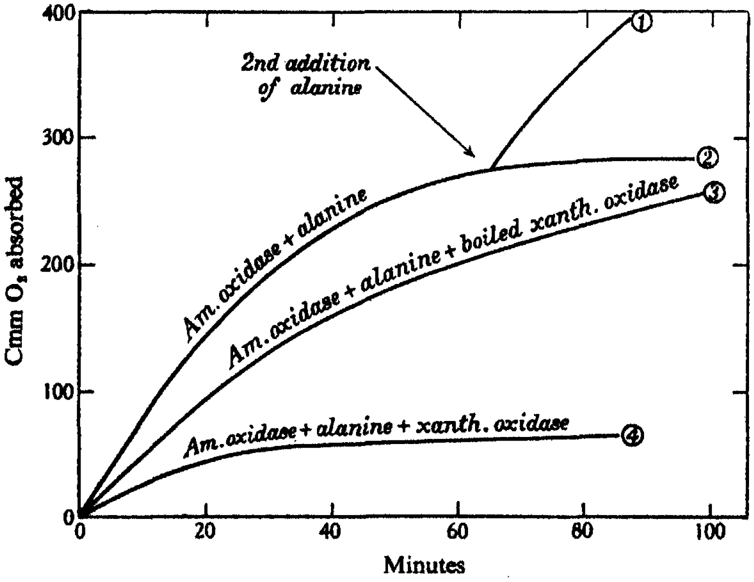


FIG. 10—Inhibition of amino acid oxidase + *dl*-alanine by xanthine oxidase. Effect of second addition of *dl*-alanine.

### 5—*Formation of $H_2O_2$*

The formation of hydrogen peroxide during oxidations of amino acids was more difficult to detect than in the previously described oxidation of uric acid. *p*-phenylenediamine and other similar compounds cannot be used for detection of hydrogen peroxide because they are powerful inhibitors of the enzyme. With some preparations we have obtained distinct positive results in using cerium hydroxide, but unfortunately with other preparations this method gave almost negative results.

---

* Unpublished observation in collaboration with Dixon.

That hydrogen peroxide is formed during the primary reaction was, however, clearly demonstrated when using alcohol as a substrate for the secondary oxidation. This oxidation reaction will be described at length in the following paper.

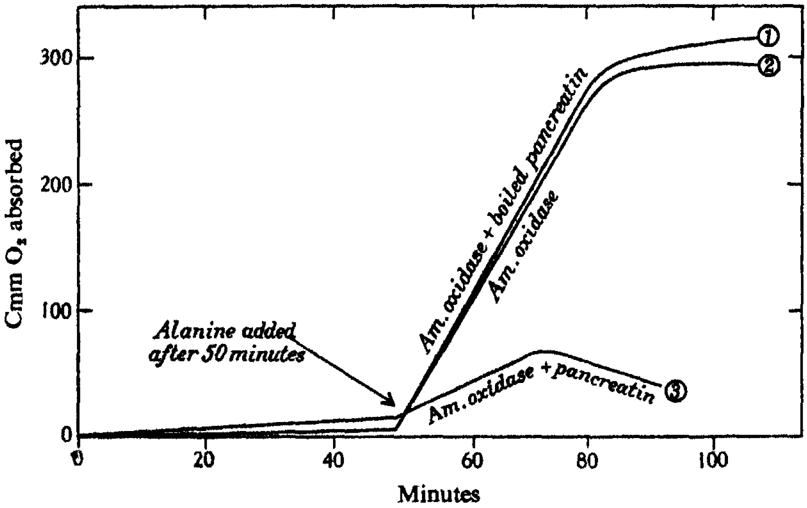


FIG. 11—Inhibition of amino acid oxidase by pancreatin.

## 6—*Specificity of the Enzyme and Oxidation of Methylated Amino Acids*

The enzyme, as has already been shown by Krebs, oxidizes only the unnatural amino acids belonging to the *d*-group. We have been able to confirm his statement, and we have found that natural amino acids when added to the enzyme, even in high concentration, do not interfere with the oxidation of unnatural acids; for instance, the velocity of oxidation by the enzyme of *l*-alanine (5 mg)* is not affected by the addition of 40 mg of *d*-alanine. This shows that not only is the enzyme incapable of activating the compounds of the *l*-group but that it does not even form complexes with them. It was interesting, however, to investigate the behaviour of methylated amino acids.

---

* A sample of this compound was kindly given to us by Dr. F. B. Kipping to whom we address here our thanks.

For this purpose, we have prepared three compounds,

N-monomethylalanine ($CH_3$ CH $NHCH_3$ COOH),
N-dimethylalanine ($CH_3$ CH $N(CH_3)_2$ COOH), and
α-methylalanine ($(CH_3)_2$ $CNH_2$ COOH).

The first two were synthesized according to Friedmann's method (1908) from α-bromopropionic acid and mono- or dimethylamine. The third compound was prepared, according to Zelinsky and Stadnikoff's method (1906), from acetone condensed with sodium cyanide and ammonium chloride to give α-amino *iso*-butyronitrile which was hydrolysed by hot hydrochloric acid. The compound was then purified in the usual way with silver oxide and hydrogen sulphide. Of these three compounds we found that the enzyme was capable of oxidizing only the N-monomethylalanine, fig. 12. The two others did not undergo oxidation and did not interfere with the normal oxidation of *l*-alanine, or of N-monomethylalanine. The oxidation of N-monomethylalanine leads to the formation of pyruvic acid and the liberation of methylamine. The latter can be easily recognized by converting it into methylcarbylamine by warming the solution with chloroform and strong alkali.

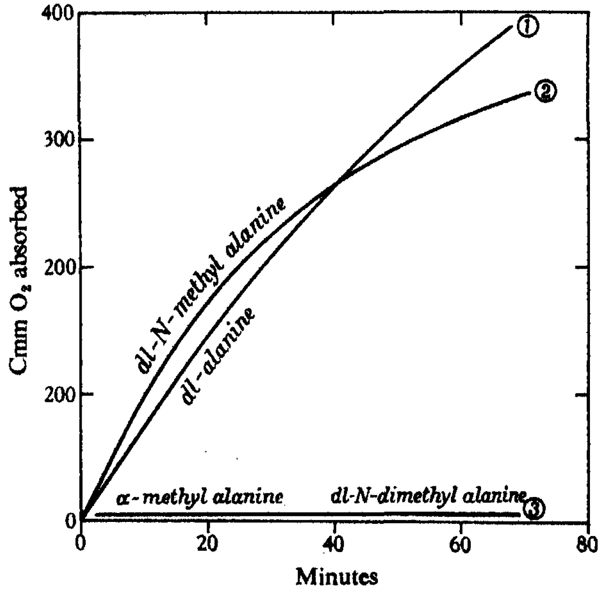


FIG. 12—Oxidation of substituted alanines, 5 mg, by amino acid oxidase.

We have also tested as a substrate for this enzyme *dl*- N-monomethyltyrosine (synthetic surinamine)* and found that the enzyme does not catalyse the oxidation of this compound. This unexpected result shows that while the presence of the parahydroxy ring does not interfere with the catalytic oxidative deamination of tyrosine itself, its presence in N-monomethyltyrosine prevents the demethylamination of this compound.

### 6—*Oxidation of Tyrosine and Dioxyphenylalanine*

A clear extract of amino acid oxidase catalyses the oxidative deamination of *d*-tyrosine and the *d*-component of *dl*-dioxyphenylalanine (dopa) to the corresponding keto acids and this reaction is not inhibited by cyanide. On the other hand, kidney slices or fresh kidney tissue pounded with sand and suspended in buffer solution ($p_H$ 7·8) can, in addition to this oxidative deamination reaction, promote also the oxidation of both optical isomers of dioxyphenylalanine, though not of tyrosine, by attacking the ring portions of their molecules. This oxidation reaction which is inhibited by cyanide, leads to the formation of a black pigment. It is promoted by a polyphenol oxidase present in the preparation which oxidizes also catechol, pyrogallol, and adrenaline, but not the monophenols such as phenol, paracresol, and tyrosine. The same enzyme will probably oxidize the dioxyphenylpyruvic acid obtained by deamination of the "*d*-dopa" by the soluble amino acid oxidase. We could not, however, demonstrate this reaction experimentally, as it requires the unnatural optical isomer "*d*-dopa," which we have not yet succeeded in preparing. We can say, however, that such a reaction would be analogous to the oxidation by tyrosinase of parahydroxyphenylpyruvic acid, obtained by the deamination of *d*-tyrosine by amino acid oxidase. That this reaction takes place can be demonstrated in the following way: 5 mg of *d*-tyrosine are oxidized in a Barcroft differential manometer by a purified preparation of amino acid oxidase. The oxidation comes to an end when the oxygen uptake reaches the value which corresponds approximately to one atom of oxygen per molecule of amino acid. At this stage the taps of the manometer are opened, and a tube with 10 mg of tyrosinase preparation is suspended from the potash tube of the flask. After equilibration of the temperature, the taps are closed, the tube is displaced, mixing its contents with that of the flask, after which readings are again taken. As fig. 13 shows, after the first reaction of oxidation promoted by amino acid oxidase is completed and the tyrosine is deaminated, a second reaction due to tyrosinase starts and gradually comes to completion.

---

* A pure sample of this compound was kindly given to us by Professor E. Winterstein to whom we address here our thanks.

The oxidation by tyrosinase of the *p*-hydroxyphenylpuruvic acid formed in the first reaction is not accompanied by the formation of the insoluble black pigment. The solution only becomes dark brown as when synthetic *p*-hydroxyphenylpyruvic acid is oxidized by tyrosinase. This acid was prepared by alkaline hydrolysis of the condensation product of *p*-hydroxybenzaldehyde and hippuric acid (Plöchl, 1883). The formation only of the brown colour explains why this reaction of oxidation was overlooked by Raper and Wormall (1925) who relied in their investigations only on the appearance of the insoluble pigment.

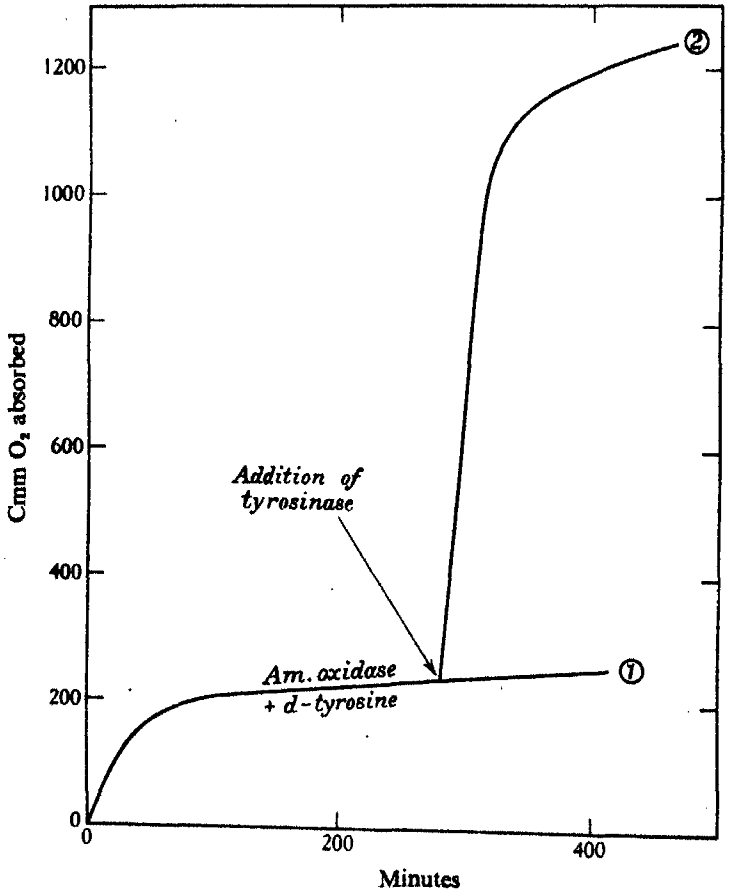


Fig. 13—Oxidation by tyrosinase of deaminated *d*-tyrosine, 5 mg.

### 8—*Behaviour of the Enzyme in Slices and in Mixtures with other Enzymes*

It was shown recently by Krebs (1935) that the deamination of *d*- and *l*-amino acids such as alanine by tissue slices is inhibited by the addition of other substances such as lactic acid, which can be oxidized by the same tissue. In other words, if two substances which are capable of increasing the oxygen uptake of the tissue slice are added together, there is no summation of the effects which they can produce separately. He found also that while KCN has no inhibitory effect on the oxidation of the unnatural amino acid by the extracted enzyme, it inhibits this reaction in slices by 10 to 40%. Under the same conditions, the normal respiration of tissue slices is inhibited by 80 to 90%. From these interesting observations Krebs concluded that in slices the *d*-amino acid deaminase system does not behave in the same way as it does in extracts. While in extracts it reacts with molecular oxygen, in intact slices it competes with other systems for the " activated oxygen," which he claims explains the partial inhibition by KCN and the absence of summation effect. In other words, the properties of an enzyme in extracts and in intact slices are very different. This interesting interpretation, although very plausible at first sight, does not explain other observations in the same category. It is obvious that the activated molecules of substrates which can undergo oxidation only by reacting with the oxidase-cytochrome system, or with an autoxidizable carrier, will naturally compete for this system or carrier. As long as the cell is not saturated with such activated molecules, the effects produced by their addition will be additive. When, however, the systems reacting with oxygen are completely saturated with activated molecules, or, in other words, when the velocity of their reactions with oxygen becomes the limiting factor of respiration, the addition of other metabolites will have no effect on respiration. This explains the absence of summation of effects produced by the simultaneous addition of different metabolites to yeast cells. It cannot explain, however, the inhibition of oxidative deamination of non-natural alanine in kidney slices produced by the addition of lactic acid, because we have no evidence that activated alanine can react with either an autoxidizable carrier or a system similar to oxidase-cytochrome. It seems to us that the enzymes such as amino acid oxidase and uricase, having a comparatively low affinity for oxygen, cannot compete favourably in tissue slices for molecular oxygen with Warburg's system, which has a high affinity for oxygen. Moreover, 10 to 40% inhibition by KCN in concentrations which inhibit the respiration of the cells by 80 to 90% gives no indication that the *d*-amino acid deaminase system reacts with "activated oxygen." The failure to produce summation of effects by the addition of two substrates and a more or less distinct depression by KCN of the activity of an enzyme which is usually resistant to cyanide, cannot be considered as a property peculiar only to intact cells or tissue slices. In fact, similar conditions can be realized in mixtures of different enzyme preparations obtained from pounded tissue, dried acetone cells, or even cell-free enzyme preparations.

TABLE I

The oxidation of uric acid, 10 mg, and *dl*-alanine, 10 mg, by a mixture of uricase, 50 mg, and amino acid oxidase, 50 mg.

Effect of M/250 KCN. T = 39° C. $p_H$ = 7·8.

| Uricase | Amino acid oxidase | Uric acid | Alanine | KCN | Cmm $O_2$ per hour |
|---|---|---|---|---|---|
| + | + | + | — | — | 245 |
| + | + | — | + | — | 465 |
| + | + | + | + | — | 465 |
| + | + | + | — | + | 24 |
| + | + | — | + | + | 471 |
| + | + | + | + | + | 360 |

As is shown in Table I, the addition of uric acid and *d*-alanine together to the mixture of two enzymes, uricase and amino acid oxidase, does not produce the summation of the effects which can be obtained by each substrate added separately. Moreover, the cyanide which strongly inhibits uricase and has no effect on amino oxidase, depresses the activity of the latter, when added to the mixture of both systems, by at least 30%.

Similar results with regard to the summation can also be obtained by combining succindehydrogenase and amino acid oxidase, Table II.

TABLE II

The oxidation of succinic acid, 9 mg sodium salt, and *dl*-alanine, 10 mg, by a mixture of amino acid oxidase, 50 mg, and succinoxidase, from 100 mg heart muscle. T = 39° C. $p_H$ = 7·8.

| Succin oxidase | Amino acid oxidase | Succinic acid | Alanine | Cmm $O_2$ per hour |
|---|---|---|---|---|
| + | + | + | — | 492 |
| + | + | — | + | 336 |
| + | + | + | + | 435 |

An interesting effect is also produced when two enzymes such as tyrosinase and amino acid oxidase act simultaneously on the same substance such as *d*-tyrosine, Table III. In this case, amino acid oxidase deaminates tyrosine, while tyrosinase oxidizes the unmodified tyrosine to melanine and the deaminated tyrosine to an unknown compound (*see* p. 134). While the amino acid oxidase is not inhibited by cyanide, the tyrosinase is very strongly inhibited by it.

TABLE III

The simultaneous oxidation of *d*-tyrosine, 5 mg, by tyrosinase, 50 mg, and amino acid oxidase, 50 mg.

Effect of M/250 KCN. T = 39° C. $p_H = 7 \cdot 8$.

| Tyrosinase | Amino acid oxidase | Tyrosine | KCN | Cmm $O_2$ per hour |
|---|---|---|---|---|
| + | – | + | – | 183 |
| – | + | + | – | 197 |
| + | + | + | – | 176 |
| + | – | + | + | 5 |
| – | + | + | + | 210 |
| + | + | + | + | 115 |

## IV—XANTHINE OXIDASE OR SCHARDINGER ENZYME

This enzyme was the subject of very extensive investigations by Hopkins, Dixon, and Wieland, with their co-workers. Its properties have been well established and the kinetics of its action have been well worked out. The enzyme catalyses the oxidation of purines such as hypoxanthine and xanthine to uric acid and aldehydes to the corresponding acids. Wieland and his co-workers have, however, recently claimed to have demonstrated that the oxidations of purines and of aldehydes are catalysed by two distinct enzymes—xanthine oxidase and Schardinger enzyme respectively. Although it seems somewhat surprising that the same enzyme should catalyse the oxidation of substances so different as purines and aldehydes, careful study of this enzyme by Dixon and Thurlow (1924) and more recently by Booth (1935) has definitely shown that we are dealing here with only one enzyme. In fact, both properties, the oxidation of purines and of aldehydes, are always found together in enzyme preparations of different origin and obtained by different methods. Any treatment of the enzyme or the addition of any reagent which inhibits the oxidation of purines inhibits also the oxidation of aldehydes. Moreover, uric acid acts as a competitive inhibitor in the oxidation of both groups of substances (Dixon and Thurlow, 1924). Finally, there is a definite competition and not addition, when both types of substrate are added simultaneously (Booth, 1935). All this clearly demonstrates the identity of xanthine oxidase and Schardinger enzyme.

Both methylene blue and oxygen can act equally well as hydrogen acceptors (Green and Dixon, 1934). When the activated substrate reacts with oxygen, the latter becomes reduced to hydrogen peroxide (Thurlow, 1925), which can be used in the secondary oxidation reaction. The enzyme is not inhibited by CO (Dixon, 1927) and $H_2S$ but is irreversibly inhibited by cyanide only when the latter is incubated with the enzyme before the addition of the substrate.* If, however, the enzyme is mixed with the substrate before the addition of the cyanide, the latter does not inhibit the activity of the enzyme.

It was shown by previous workers that the primary oxidation system, especially with aldehydes as substrate, may not give the theoretical oxygen uptake and that the reaction usually stops long before the oxidation of the substrate is completed. This incomplete oxidation of substrate, as was shown by Dixon (1925), is due to the destruction of the enzyme by hydrogen peroxide formed during the reaction. The reaction can, however, reach completion when the enzyme is protected from destruction by hydrogen peroxide, and this can be obtained either on addition of catalase (Dixon, 1925) or of cerium hydroxide (Wieland and Rosenfeld, 1930). We have confirmed these observations and found that the addition to this system of catalase preparation free from alcohol brings the oxygen uptake to the theoretical amount required for the oxidation of hypoxanthine to uric acid or of aldehyde to the corresponding acid.

The enzyme shows a peculiar specificity. Although it oxidizes aldehydes and purines, among the latter it activates only hypoxanthine, xanthine, 6:8-dioxypurine, and to some extent adenine. It does not catalyse the oxidation of any other purines.

On the other hand, some of these substances, like uric acid, 1-methyl xanthine, 3-methylxanthine, guanine, etc., act as very strong competitive inhibitors which shows that they combine with the same active grouping of the enzyme as hypoxanthine or aldehydes themselves (Coombs, 1927).

## V—SUMMARY AND CONCLUSIONS

*Uricase* is a highly specific enzyme and catalyses only the oxidation of uric acid to allantoin. It does not oxidize any of the mono-, di-, or trimethylated or ethylated derivatives of uric acid.

These compounds, however, inhibit the oxidation of uric acid, which shows that they react with the same active grouping of the enzyme molecule as uric acid itself and act therefore as competitive inhibitors.

---

* Unpublished observation in collaboration with Dr. Dixon.

The oxidation of uric acid catalysed by this enzyme takes place only aerobically, and for each molecule of uric acid oxidized, one molecule of oxygen is reduced to hydrogen peroxide. The reaction proceeds according to the following equation:

$$\text{Uric acid} + 2H_2O + O_2 = \text{allantoin} + CO_2 + H_2O_2;$$

the $H_2O_2$ being either decomposed by catalase, liberating oxygen, or used in the oxidation of another molecules of uric acid.

*Amino acid oxidase*, or *d*-amino acid deaminase of Krebs, catalyses oxidative deamination of non-physiological optically active α-amino acids (belonging to *d* group) to the corresponding keto acids.

This enzyme catalyses also the oxidation of N-monomethylalanine ($CH_3\,CH\,NH\,(CH_3)\,COOH$) to the corresponding keto acid with the liberation of a molecule of methylamine.

It does not catalyse, however, the oxidation of N-dimethylalanine, α-methylalanine, and N-monomethyltyrosine.

There is no evidence that the mechanisms of oxidation of *d*-amino acid by the extracted amino acid oxidase and by the tissue slices are different.

When the oxidation takes place in presence of oxygen, for each molecule of amino acid oxidized, one molecule of oxygen is reduced to $H_2O_2$.

Comparing these two enzymes with xanthine oxidase, it can be seen that they all behave as true dehydrogenases, activating their corresponding substrates. The activated substrate, in xanthine oxidase, can react equally well with methylene blue and oxygen, while in amino acid oxidase it reacts with methylene blue fifty times more slowly than with oxygen, and in uricase reacts only with oxygen.

Cyanide in very low concentrations produces instantaneous strong but reversible inhibition of uricase, strong, irreversible inhibition of xanthine oxidase when incubated with the enzyme in absence of substrate, and no inhibition of amino acid oxidase.

$H_2S$ has no effect on uricase and xanthine oxidase but produces strong irreversible inhibitions of amino acid oxidase when incubated with the enzyme in presence of oxygen.

CO, on the other hand, has no effect on either of these enzymes.

We do not yet know whether the activity of the enzymes described in this paper requires the co-operation of co-enzymes or whether the activated molecules of substrates require for their oxidation the presence of a carrier. We can say, however, that with xanthine oxidase, various methods of preparation and of purification by precipitations, absorptions, and elutions, ultrafiltration and electrodialysis, so far, have not revealed the presence of either a co-enzyme or of a carrier. This does not apply, however, to uricase and especially to amino acid oxidase, which in this respect have not yet been sufficiently investigated. It is possible that co-enzymes analogous to those which co-operate with other dehydrogenases are also present in these enzyme preparations but being more intimately combined with the enzymes, are more difficult to detect by the usual methods.

## REFERENCES

Bach, A. (1914). 'Biochem. Z.,' vol. **60**, p. 221.
Battelli, F., and Stern, L. (1909). 'Biochem. Z.,' vol. **19**, p. 219.
—— (1912). 'Ergebn. Physiol.,' vol. **12**, p. 199.
Bernheim, F., and Bernheim, M. (1934). 'J. Biol. Chem.,' vol. **107**, p. 275.
Biltz, H., and Schauder, H. (1923). 'J. prakt. Chem.,' vol. **106**, p. 108.
Booth, V. H. (1935). 'Biochem. J.,' vol. **20**, p. 1731.
Brüning, H., Einecke, F., Peters, F., Rabl, R., and Viehl, K. (1928). 'Z. physiol. Chem.,' vol. **174**, p. 94.
Coombs, H. I. (1927). 'Biochem. J.,' vol. **21**, p. 1259.
Dixon, M. (1925). 'Biochem. J.,' vol. **19**, p. 507.
—— (1927). 'Biochem. J.,' vol. **21**, p. 1211.
Dixon, M., and Thurlow, S. (1924). 'Biochem. J.,' vol. **18**, p. 976.
Felix, K., Scheel, F., and Schuler, W. (1929). 'Z. physiol. Chem.,' vol. **180**, p. 90.
Foss, *et al.* (1929). 'C.R. Acad. Sci. Paris,' vol. **189**, p. 213.
—— (1930, *a*). *Ibid.*, vol. **190**, p. 79.
—— (1930, *b*). *Ibid.*, vol. **191**, p. 1153.
Friedmann, E. (1908). 'Beitr. chem. Physiol. Path.,' vol. **11**, pp. 164, 195.
Green, D., and Dixon, M. (1934). 'Biochem. J.,' vol. **28**, p. 237.
Grynberg, M. Z. (1931). 'Biochem. Z.,' vol. **236**, p. 138.
Harrison, D. C. (1929). 'Biochem. J.,' vol. **23**, p. 982.
Harrison, D. C., and Thurlow, S. (1926). 'Biochem. J.,' vol. **20**, p. 217.
Kisch, B. (1931). 'Biochem. Z.,' vol. **237**, p. 226; vol. **238**, p. 351.
Krebs, H. A. (1933). 'Z. physiol. Chem.,' vol. **217**, p. 351.
—— (1935). 'Biochem. J.,' vol. **29**, p. 1620.
Plöchl, J. (1883). 'Ber. deuts. chem. Ges.,' vol. **16**, p. 2815.
Przylęcki, St. J. (1925, 1926). 'Arch. Int. Physiol.,' vol. **24**, p. 237; vol. **27**, p. 159.
Pryzlęcki, St. J., and Truszkowski (1928). 'C.R. Soc. Biol. Paris,' vol. **98**, p. 789.
Raper, H. S., and Wormall, A. (1925). 'Biochem. J.,' vol. **19**, p. 84.
Remesow (1932). 'Biochem. Z.,' vol. **246**, p. 431.
Ro, K. (1931). 'J. Biochem. (Japan),' vol. **14**, p. 361.
Schittenhelm, A. (1905). 'Z. physiol. Chem.,' vol. **46**, p. 354.
Schuler, W. (1932). 'Z. physiol. Chem.,' vol. **208**, p. 237.
Thurlow, S. (1925). 'Biochem. J.,' vol. **19**, p. 175.
Truszkowski (1930). 'Biochem. J.,' vol. **24**, p. 1340.
Wiechowski and Wiener (1909). 'Arch. exp. Path. Pharmak.,' vol, **60**, p. 185.
Wieland and Rosenfeld (1930). 'Liebig's Ann.,' vol. **477**, p. 32.
Zelinsky, N., and Stadnikoff, G. (1906). 'Ber. deuts. chem. Ges.,' vol. **39**, p. 1722.

577 · 158 · 432

# Coupled Oxidation of Alcohol

By D. KEILIN, F.R.S., and E. F. HARTREE, Ph.D.

(From the Molteno Institute, University of Cambridge)

(*Received October* 21, 1935)

## I—INTRODUCTION

We have seen previously that hydrogen peroxide is formed in several oxidation reactions catalysed by enzymes like xanthine oxidase, uricase, and amino acid oxidase. In all these reactions one molecule of hydrogen peroxide is formed for each molecule of substrate oxidized. It is known that the hydrogen peroxide formed in the primary oxidation reaction can be used in promoting secondary or coupled oxidations of some substances (Thurlow, 1925; Harrison and Thurlow, 1926). The study of secondary oxidations by hydrogen peroxide revealed an unsuspected fact, namely, that it can be used in the oxidation of alcohol to the corresponding aldehydes. In this paper we propose to examine the conditions under which the coupled oxidation of alcohol takes place, the properties of the peroxide formed in the primary reaction, and the mechanism of the secondary oxidation of alcohol promoted by this peroxide.

## II—COUPLED OXIDATION OF ALCOHOL BY URICASE SYSTEM

The study of this reaction was carried out in Barcroft differential manometers in the usual manner but with a series of controls. For instance, in order to detect the oxidation of alcohol by the uricase system, the oxygen uptake of four mixtures was measured separately: (1) uricase + uric acid; (2) uricase + alcohol; (3) uricase + uric acid + alcohol; (4) uric acid + alcohol. The reactions were carried out in buffer solution of $p_H$ 8·9 at 39° C. The quantity of enzyme varied from 25 to 50 mg of dry acetone preparation of liver, that of uric acid varied from 2 to 5 mg, and of alcohol from 5 to 10 mg per flask. The $CO_2$ was absorbed in the usual way by rolls of filter paper soaked with 10% KOH. The substrate in these experiments was always added in small dangling tubes suspended from the KOH tubes. These tubes were dislodged and their contents mixed with that of the flask after equilibration of the temperature and closing the taps of the manometers. After the experiment the contents of the flasks were examined for aldehydes. In manometric experiments with uricase the presence of KOH is required for absorption of $CO_2$, and most of the aldehyde is absorbed by the potash paper where it polymerizes to a dark brown substance. In this way, even a small amount of aldehyde formed from alcohol gives rise to a yellow fringe on the potash filter paper.

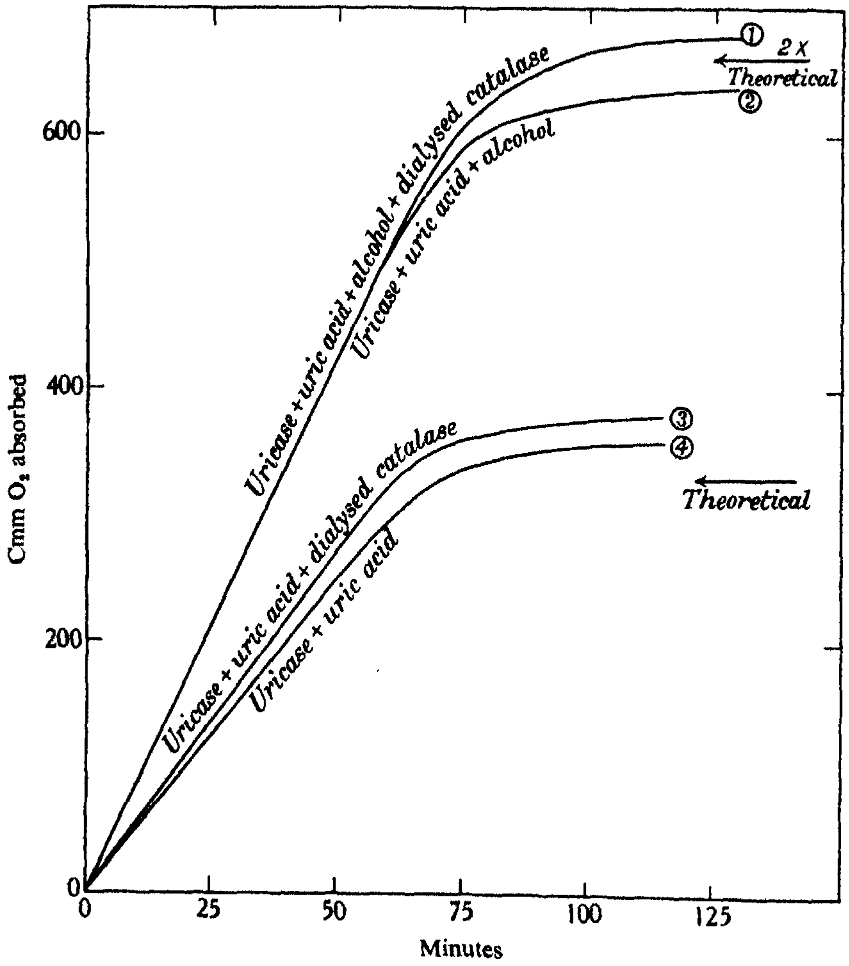


Fig. 1—Coupled oxidation of ethyl alcohol, 10 mg, by uricase + uric acid, 5 mg, at 39° C.

Uricase + catalase + alcohol }
Uricase + alcohol } No oxidation.

As fig. 1 shows, while uricase + alcohol does not show any appreciable absorption of oxygen, uricase + uric acid takes up just a little more than the theoretical amount necessary for oxidation of uric acid to allantoin, and uricase + uric acid + alcohol takes up twice this theoretical amount. The experiment was repeated several times with different uricase preparations and always gave the same result. Of other alcohols tried, methyl alcohol undergoes similar oxidation, *n*-propyl alcohol oxidizes rapidly but does not double the theoretical oxygen uptake, while amyl alcohol does not oxidize at all, fig. 2. The addition of catalase preparation to this system has very little effect on the reaction, because uricase preparation itself contains much catalase. We have found, however, that even when added in great excess, catalase did not remove the hydrogen

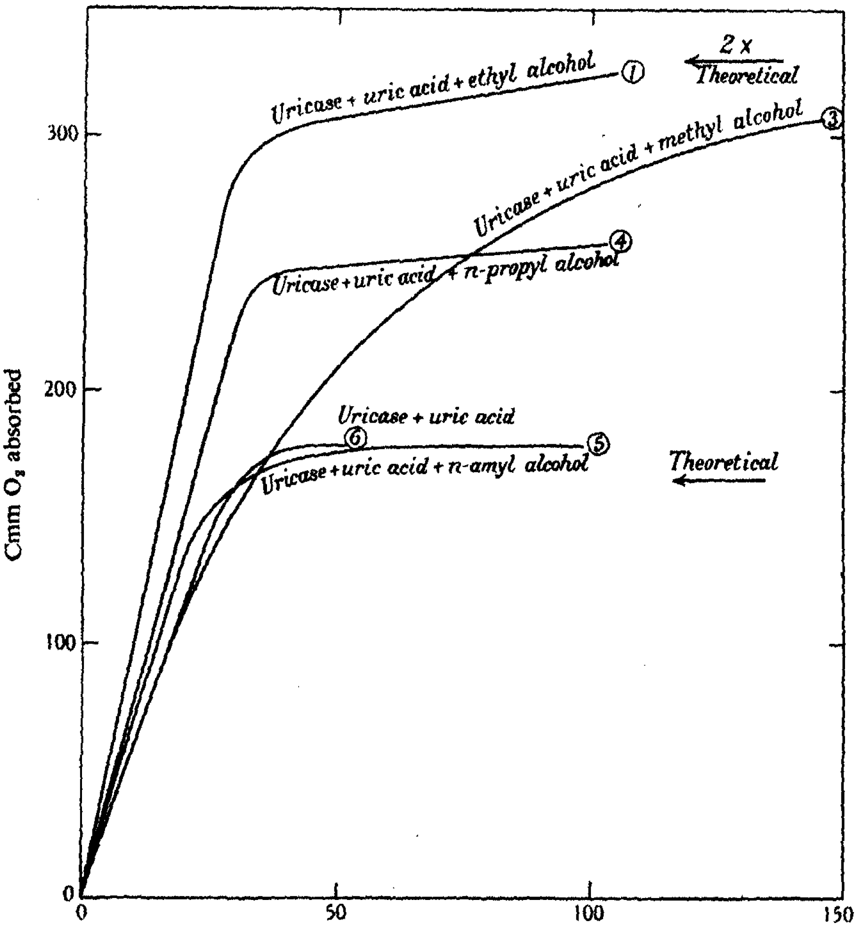


Fig. 2—Coupled oxidation of alcohols, 10 mg, by uricase.

peroxide by splitting it in the usual way and did not therefore interfere with the secondary oxidation of alcohol. The fact that catalase did not remove $H_2O_2$ formed in the primary reaction may be due to its having a much lower affinity for $H_2O_2$ compared with that of the secondary oxidation system.

## III—COUPLED OXIDATION OF ALCOHOL BY *d*-AMINO ACID DEAMINASE SYSTEM

Although it was difficult to detect in the usual way, the formation of hydrogen peroxide during the oxidation of unnatural amino acids by *d*-amino acid deaminase, the addition of alcohol to this system invariably gave distinctly positive results. The experiments for this purpose were carried out with Barcroft differential manometers in the same way as those with uricase system with the exception that the presence of KOH was found unnecessary, $CO_2$ not being formed in the primary reaction. In these experiments, the amount of enzyme used per flask corresponded to an extract of 50 mg of acetone powder preparation. That of amino acid (*dl*-alanine or *dl*-valine) varied from 5 to 10 mg added from the dangling tubes after closing the taps of the manometer. In these experiments the mixtures of enzyme and alcohol or of amino acids and alcohol had no appreciable uptake of oxygen. The mixture of enzyme and amino acid took up a theoretical amount of oxygen corresponding to one atom of oxygen per molecule of amino acid oxidized, while the mixture of enzyme, amino acid, and alcohol took up nearly twice this amount, fig. 3. The fact that the amino acid oxidase preparation is fairly rich in catalase shows already that the latter, as in the case of uricase, does not destroy the hydrogen peroxide formed in the primary reaction and therefore does not inhibit the secondary oxidation of alcohol. The addition of catalase increases the oxygen uptake to exactly double that of the primary oxidation reaction.

## IV—COUPLED OXIDATION OF ALCOHOL BY XANTHINE OXIDASE SYSTEM

### *a—Oxidation of Alcohol in Presence of Catalase Preparation*

In order to see whether alcohol undergoes a secondary oxidation in the presence of this system, we have repeated with it a series of experiments similar to those which were carried out with uricase and amino acid oxidase. For this purpose we have measured the velocities and the total oxygen uptake by xanthine oxidase and its various substrates (hypoxanthine and different aldehydes) with and without the addition of alcohol.

Xanthine oxidase was prepared from milk as caseinogen or as whey preparation and was usually defatted with ether and kept dry. According to the age of the preparation, 25 to 100 mg of dry weight of enzyme was used per flask with an amount of substrate which varied for hypoxanthine from 1 mg to 2 mg, for freshly redistilled acetaldehyde from 0·4 to 1·5 mg, and for alcohol 5 to 10 mg.

The results of the first series of experiments have clearly shown that the addition of alcohol to xanthine oxidase + hypoxanthine or xanthine oxidase + acetaldehyde does not increase the total oxygen uptake by these systems, which shows that the hydrogen peroxide formed during the primary oxidation reaction does not promote the oxidation of alcohol. Even on addition of strong peroxidase preparation to the system alcohol does not undergo oxidation.

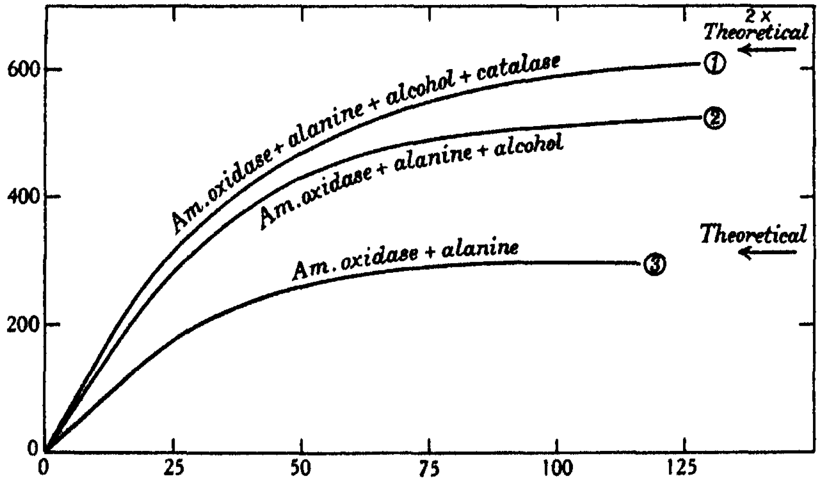


FIG. 3—Coupled oxidation of alcohol by amino acid oxidase.

It was, however, a matter of some surprise to us to find that on addition of catalase preparation to the system of xanthine oxidase + hypoxanthine + alcohol, the latter undergoes oxidation to aldehyde and doubles the oxygen uptake of the primary reaction. This result was unexpected because catalase is the only enzyme which by its property of rapid decomposition of $H_2O_2$ was expected to abolish and not to promote the secondary oxidation reaction by $H_2O_2$ formed in the primary reaction.

*The catalase preparation* used in these reactions was obtained from horse liver by the method of Zeile and Hellström (1930) slightly modified which gives a strong solution of catalase, containing, however, varying concentrations of alcohol and of chloroform. This strong solution was purified either by electrodialysis against distilled water or by ultrafiltration. The latter was carried out with graded Bechhold's ultra filter membranes supported by a perforated porcelain disc and clamped in a special porcelain funnel fixed into a vacuum flask. The catalase solution was poured on to the membrane impermeable to catalase, the surface of the membrane being constantly swept by a fine brush driven by a small electric motor. The filtration was carried out under a negative pressure, the enzyme being constantly washed by the addition of fresh distilled water. In addition to catalase from liver, we have used also a haemoglobin free catalase preparation obtained from blood by Tsuchihashi's method (1930) modified in the following way: Ox or horse blood is defibrinated, washed in saline, laked with distilled water, acidified to $p_H$ 6, and treated with the minimum amount of potassium ferricyanide to oxidize all the haemoglobin to methaemoglobin. After removing the ferricyanide by electrodialysis, 100 cc of methaemoglobin solution thus obtained is mixed with 10 cc of chloroform and vigorously shaken for 5 minutes. The methaemoglobin rapidly becomes denatured and precipitates as cathaemoglobin. The mixture is centrifuged and a yellow solution rich in catalase but completely free from haemoglobin is thus obtained. This solution is washed and concentrated by the previously described methods of ultrafiltration. For some experiments, we have used also as catalase diluted laked human blood or a preparation obtained from cucumber seeds by the method described by Zeile (1930).

A large number of experiments has been carried out with different xanthine oxidase preparations, different concentrations of hypoxanthine and of aldehyde with and without the addition of catalase and of alcohol. Various preparations of catalase were added either in crude form, containing alcohol, or dialysed and alcohol free. The results of these experiments, which are summarized in Table I and fig. 4, clearly show that in the presence of the primary reaction of oxidation of hypoxanthine or of aldehyde by xanthine oxidase, alcohol does not undergo oxidation. It is only when catalase preparation is added to the system that alcohol is oxidized to aldehyde, with the result that the total oxygen uptake of the system becomes double or even more than double that of the primary oxidation.

### *b—Cyclic Oxidation*

In some of the experiments, in the absence of KOH and especially when the substrate of the primary reaction is an aldehyde, the $H_2O_2$ formed in this reaction, when used in the secondary oxidation of alcohol to aldehyde, increases the supply of substrate for the primary reaction.

There is, therefore, no reason for this reaction to come to an end while the alcohol is still present in solution and the enzymes involved in the reaction are still intact, figs. 5 and 6. In this reaction, the oxidation proceeds far beyond doubling or even tripling of the primary oxygen uptake. Such reaction of oxidation, which we propose to call " cyclic oxidation " may, in fact, occur in liver, which contains all the enzymes and may also contain the substrates necessary for this reaction. If, on the other hand, the primary reaction consists in the oxidation of purines, the secondary, cyclic oxidation of alcohol does not go beyond tripling the

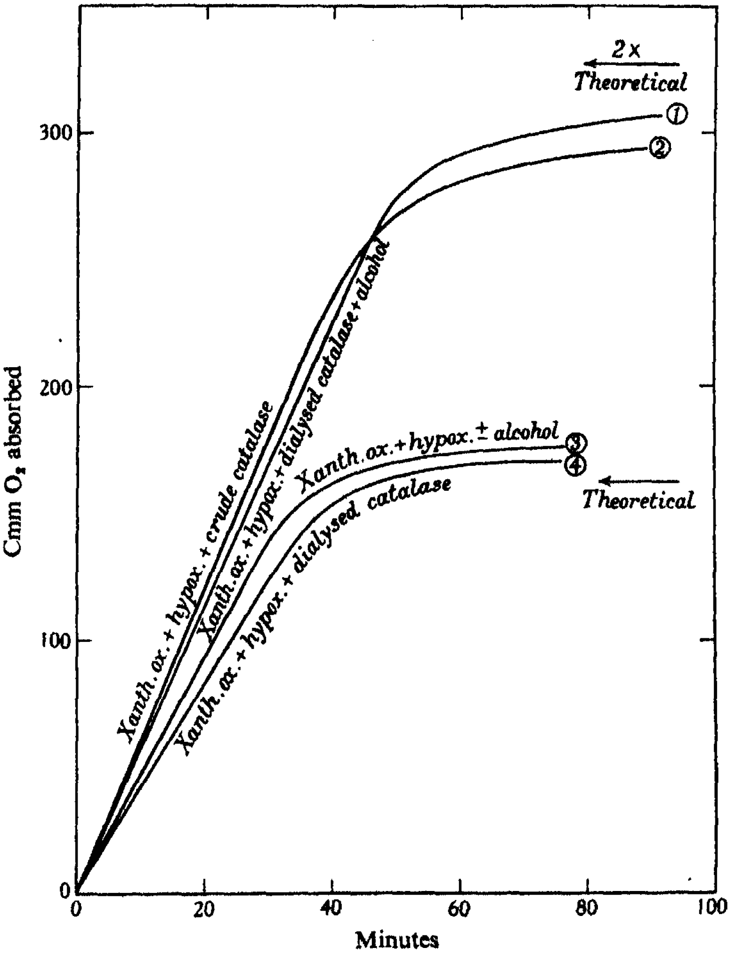


FIG. 4—Coupled oxidation of alcohol by xanthine oxidase (100 mg caseinogen preparation) + hypoxanthine (1 mg) + catalase. KOH papers used.

TABLE I

Coupled oxidation of alcohol by xanthine oxidase and hypoxanthine or aldehyde in presence of catalase preparation; theoretical uptake by 1 mg of hypoxanthine = 166 cmm, by 0·4 mg of acetaldehyde = 100 cmm. Catalase: *b*, boiled; *d*, dialysed.

| Expt. No. | Xanthine oxidase 100 mg | Hypo-xanthine 1 mg | Acetal-dehyde 0·4 mg | Catalase | Alcohol 10 mg | Cmm $O_2$ |
|---|---|---|---|---|---|---|
| 1 | + | + | – | – | – | 137 |
| | + | – | – | + | + | 0 |
| | + | + | – | +*b* | + | 142 |
| | + | + | – | – | + | 157 |
| | + | + | – | +*d* | + | 600 |
| 2 | + | + | – | – | – | 167 |
| | + | + | – | – | + | 105 |
| | + | + | – | +*d* | + | 326 |
| 3 | + | + | – | – | – | 125 |
| | + | + | – | – | + | 140 |
| | + | + | – | +*b* | + | 132 |
| | + | + | – | +*d* | – | 185 |
| | + | + | – | +*d* | + | 325 |
| 4 | + | – | + | – | – | 50 |
| | + | – | + | – | + | 50 |
| | + | – | + | + | – | 95 |
| | + | – | + | + | + | >350 |
| | + | – | – | + | + | 0 |
| | + | – | – | – | + | 0 |
| | – | – | + | + | + | 0 |

oxygen uptake of the primary reaction. This may be due to the competitive inhibition of the enzyme by uric acid.

If we now bring together xanthine oxidase, uricase, hypoxanthine, and alcohol, the following reactions will take place: hypoxanthine will be oxidized by xanthine oxidase to uric acid, which in its turn will be oxidized by uricase to allantoin, and in the absence of alcohol, the reaction would come to an end when the total oxygen uptake reached the theoretical amount required for oxidation of hypoxanthine to allantoin. On addition of alcohol, the latter becomes oxidized to aldehyde which forms the substrate for xanthine oxidase and oxidizes further to the corresponding acid, the oxidation reaction thus becoming gradually cyclic. Similar results are obtained by bringing together uricase, uric acid xanthine oxidase, and alcohol, fig. 7.

## V—NATURE OF THE PEROXIDE FORMED IN THE PRIMARY OXIDATION REACTION

If the peroxide formed in the primary reaction is ordinary hydrogen peroxide, it should be possible to oxidize alcohol by bringing it together with hydrogen peroxide and catalase preparation. For this purpose several experiments have been carried out with Barcroft differential manometers. These experiments have shown, however, that whether alcohol was present or not the hydrogen peroxide added to catalase preparation was decomposed, liberating the theoretical amount of oxygen, and no trace of aldehyde could be detected in the flasks of the manometers. In order to imitate more closely the natural conditions,we have tried in some experiments to add the hydrogen peroxide very slowly. This was obtained by placing into the flasks of the manometers small tubes filled with a dilute solution of $H_2O_2$ and provided only with very fine capillary outlet

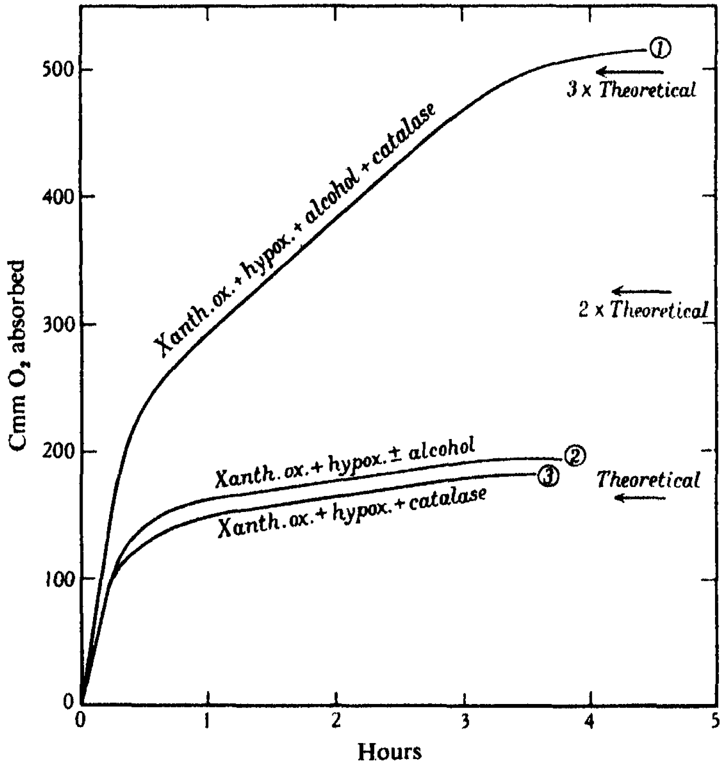


FIG. 5—Coupled oxidation of alcohol by xanthine oxidase + hypoxanthine + catalase. Cyclic reaction in absence of KOH papers.

so that $H_2O_2$ could only slowly diffuse out into the flasks. Even under these conditions, hydrogen peroxide underwent ordinary catalytic decomposition and no aldehyde could be detected within the flasks. It is only when hydrogen peroxide was replaced by some other peroxides such as

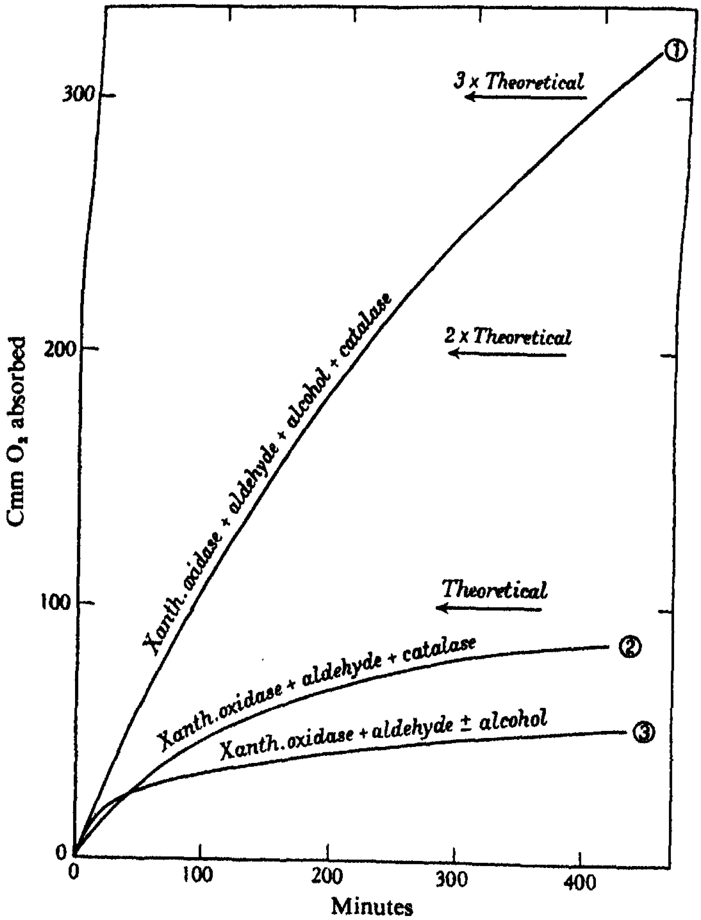


FIG. 6—Cyclic oxidation of alcohol by Schardinger enzyme system.

Aldehyde + alcohol + catalase
Xanthine oxidase + alcohol
Xanthine oxidase + alcohol + dialysed catalase
} No oxidation.

barium peroxide, cerium peroxide, and ethyl hydroperoxide that alcohol underwent oxidation to aldehyde. The easiest way to demonstrate this reaction is to shake in flasks of Barcroft manometers a mixture of alcohol, catalase, and one of these peroxides in the presence of a roll of filter paper soaked in strong KOH. The aldehyde which is formed gradually becomes absorbed by the potash paper polymerizing into the yellow to brown compound which distinctly colours the paper. The results of these experiments are summarized in Table II.

These experiments clearly show that aldehyde is only formed when catalase preparation, alcohol, and one of these peroxides are present together. They show, moreover, that catalase preparations cannot be replaced by a peroxidase preparation, a haematin compound, or a metal like palladium, and that neither of the above-mentioned peroxides can be replaced by $H_2O_2$ or by benzoyl peroxide. It must be mentioned, however, that ethyl hydroperoxide under the influence of haematin, even in the absence of alcohol, gives rise to acetaldehyde. This reaction resembles the decomposition of diethyl peroxide catalysed by ferrous salts. According to Wieland and Chrometzka (1930), in this reaction two molecules of diethyl peroxide give rise to two molecules of ethyl alcohol and two of acetaldehyde.

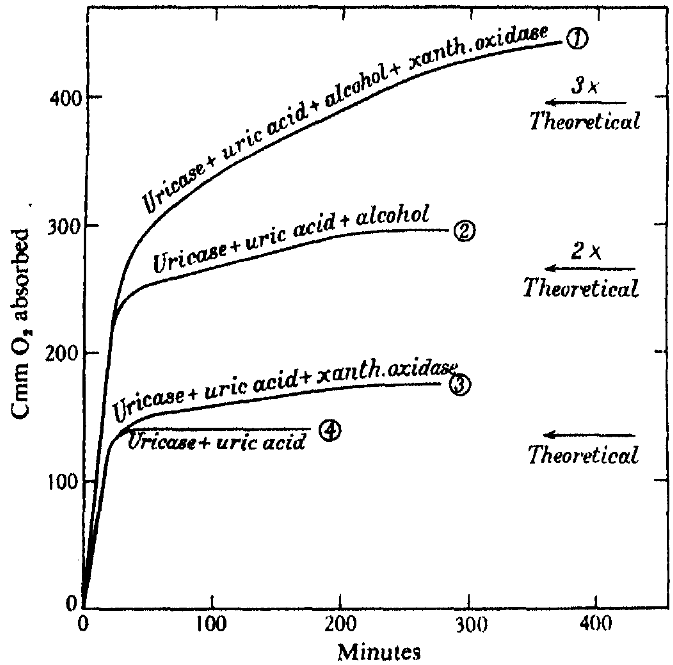


FIG. 7—Coupled oxidation of alcohol by uricase + uric acid. Cyclic oxidation induced by addition of xanthine oxidase.

On the other hand, one of the properties common to these three peroxides (barium, cerium, and ethyl) is their gradual decomposition to hydrogen peroxide. It is therefore possible that the oxidation of alcohol to aldehyde in all the above reactions is promoted by what may be called the nascent hydrogen peroxide.

We have seen also that hydrogen peroxide alone, even in the nascent stage, is not sufficient for the oxidation of alcohol and that this reaction requires the co-operation of the second factor which is present in catalase and in some other enzyme preparations.

TABLE II

| Substances added | | | | | Aldehyde formed |
|---|---|---|---|---|---|
| Catalase | Alcohol 10 mg | $BaO_2$ | Cerium peroxide 50 mg | Ethyl hydroperoxide 6·5 mg | |
| + | + | — | — | + | + |
| + | — | — | — | + | — |
| + | + | + | — | — | + |
| — | — | — | — | + | — |
| — | + | — | — | + | — |
| + | — | — | — | + | — |
| + | + | — | + | — | + |
| Peroxidase | | | | | |
| + | + | — | — | + | — |
| Haematin 0·25 mg | | | | | |
| + | + | + | | — | — |
| + | + | — | — | — | — |
| + | + | — | — | + | + |
| + | — | — | — | + | + |
| + | + | — | — | — | — |
| Palladium 1 mg | | | | | |
| + | + | + | — | — | — |
| + | + | — | + | — | — |
| Catalase | | Benzoyl peroxide 50 mg | | | |
| + | + | + | — | — | — |

## VI—MECHANISM OF SECONDARY OXIDATION OF ALCOHOL

In order to determine the constituent of catalase preparation which may be responsible for promoting the secondary oxidation of alcohol, we have tried to replace the catalase preparation by some other enzyme preparations. We have found, for instance, that the oxidation of alcohol by the xanthine oxidase system takes place also on the addition of uricase or of amino acid oxidase preparations. This explains why in both of these enzyme preparations the secondary oxidation of alcohol takes place without the addition of catalase. Both these enzyme preparations therefore shared with catalase preparation the property of catalysing the secondary oxidation of alcohol. The question that now arises is which of the constituents common to these enzyme preparations is responsible for these reactions.

*The first supposition* which it is natural to make is the presence in all these preparations of an alcohol dehydrogenase. This enzyme, known since the work of Buchner and especially of Battelli and Stern (1909–1910), is very widely distributed in animals and in plants. A large amount of work has been carried out on the properties of this enzyme within the intact cells and in cell free extracts. The most recent study of this enzyme we owe to Wieland and his co-workers, to Müller (1931 and 1934), Euler and Adler (1934), and Lehmann (1934). The results obtained by these workers are, however, often contradictory and differ with the material used for preparation of the enzyme. The activity of the enzyme in extracts is usually very low and the enzyme was found to be very fragile. In the presence only of substrate it reduces neither oxygen nor methylene blue. It is only on addition of co-zymase (Lehmann) that the methylene blue becomes reduced by this system. For the reduction of oxygen, in addition to the co-enzyme, the system requires also the presence of the yellow pigment or flavine (Euler and Adler).

We could not, however, demonstrate the presence of alcohol dehydrogenase in the enzyme preparations which strongly catalysed the secondary oxidation of alcohol. For instance, xanthine oxidase + alcohol + catalase, even on addition of co-enzyme, did not reduce methylene blue even in 2 hours, while the same system in the presence of hypoxanthine and oxygen rapidly oxidized alcohol to aldehyde. With amino acid oxidase which strongly promoted the secondary oxidation of alcohol, the rate of reduction of methylene blue, even in the presence of co-enzyme, was very slow and if anything inhibited by the addition of catalase. Moreover, catalase preparation kept in solution for over six months was still very active in catalysing the secondary oxidation of alcohol. In spite of this negative result, we cannot definitely dismiss the possibility that some kind of alcohol activating system is present in our enzyme preparations. It is conceivable, for instance, that this enzyme or system has the property of activating the molecules of alcohol only in a special way so that they can react with hydrogen peroxide formed in the primary reaction but not with other hydrogen acceptors.

*The second supposition* which is suggested by these experiments is that catalase itself may play some role in promoting the oxidation of alcohol by hydrogen peroxide. The main point which is so far in favour of this supposition is that catalatic property is an obvious property common to the three enzyme preparations (uricase, amino acid oxidase, and catalase) capable of promoting the oxidation of alcohol. This property is absent in xanthine oxidase which without the addition of catalase is incapable of catalysing the secondary oxidation of alcohol. The irreversible destruction of catalase by boiling or treating it with alkali abolishes also completely its power of catalysing the oxidation of alcohol. It was important, however, to determine the effect of some specific catalase poisons on the secondary oxidation of alcohol. It was obvious that such experiments could not be easily carried out in the presence of uricase which is inhibited by KCN and by azide. $H_2S$, on the other hand, could not be safely used as catalase poison because it can act as a substrate for oxidation induced by metals or haematin compounds present in our preparations. The effect of some of these poisons (KCN, $NaN_3$, $NH_2OH$) was tried, however, on the xanthine oxidase system with various catalase preparations added to it, and on the amino acid oxidase preparations which almost always contain catalase. The Tables III and IV and fig. 8 summarize the main results of these experiments.

TABLE III

| Xanthine oxidase 100 mg | Hypo-xanthine 1 mg | Catalase | Alcohol 10 mg | Azide m/500 | Cmm $O_2$ |
|---|---|---|---|---|---|
| + | + | + | — | — | 150 |
| + | + | + | — | + | 150 |
| + | + | + | + | — | 265 |
| + | + | + | + | + | 157 |

All these experiments show that catalase poisons such as cyanide, azide, and hydroxylamine which do not inhibit the primary oxidation reaction promoted by xanthine oxidase and by amino acid oxidase inhibit the secondary oxidation of alcohol.

*The third supposition* is that the secondary oxidation of alcohol may be due to a specific iron compound like the ferric tannate of de Stoecklin (1909) which is stated to oxidize alcohols in presence of hydrogen peroxide.

Such a compound should be present in preparations of uricase, amino acid oxidase, and catalase; but not in xanthine oxidase. Being, however, always accompanied by catalase, which rapidly decomposes ordinary $H_2O_2$, this compound will catalyse the oxidation of alcohol only in the presence of a peroxide which by its nature or its low concentration escapes the destructive decomposition by catalase.

TABLE IV

| Amino acid oxidase 50 mg | *dl*-alanine 5 mg | Alcohol 10 mg | Poison | Cmm $O_2$ | $O_2$ uptake due to alcohol |
|---|---|---|---|---|---|
| | | | KCN | | |
| + | + | — | — | 272 | — |
| + | + | + | — | 475 | 203 |
| + | + | + | M/250 | 348 | 76 |
| + | + | + | M/100 | 317 | 45 |
| + | + | — | — | 282 | — |
| + | + | — | M/500 | 272 | — |
| + | + | + | — | 475 | 193 |
| + | + | + | M/500 | 387 | 105 |
| + | + | — | — | 260 | — |
| + | + | + | — | 450 | 190 |
| + | + | + | M/500 | 345 | 85 |
| | | | $NH_2OH$ | | |
| + | + | — | — | 285 | — |
| + | + | + | — | 538 | 253 |
| + | + | + | M/1000 | 445 | 160 |
| + | + | + | M/500 | 405 | 125 |
| + | + | — | M/250 | 312 | — |
| + | + | + | M/250 | 370 | 68 |
| | | | Azide | | |
| + | + | — | — | 322 | — |
| + | + | + | — | 530 | +208 |
| + | + | — | M/100 | 450 | — |
| + | + | + | M/100 | 410 | −40 |
| + | + | — | M/500 | 382 | — |
| + | + | + | M/500 | 405 | +23 |

The catalytic activity of such an organic iron compound may also be inhibited by boiling, by treatment with alkali, and by such inhibitors as KCN, $NaN_3$, and hydroxylamine.

We must admit, however, that all attempts to separate such a compound from catalase itself, by means of dialysis, ultrafiltration, adsorption and elutions, and fractional precipitation have failed, and the extent of coupled oxidations of alcohol produced by various fractions was so far proportional to their catalatic activity. In other words, if such catalytic iron is present in our enzyme preparations it must be in combination with a protein and must have some properties in common with catalase itself.

We can mention also that we have failed to oxidize alcohol to aldehyde in presence of ferric tannate in experiments carried out under the same condition as those with catalase. Furthermore, the coupled oxidation of alcohol was not promoted by ferric tannate in presence of the xanthine oxidase system.

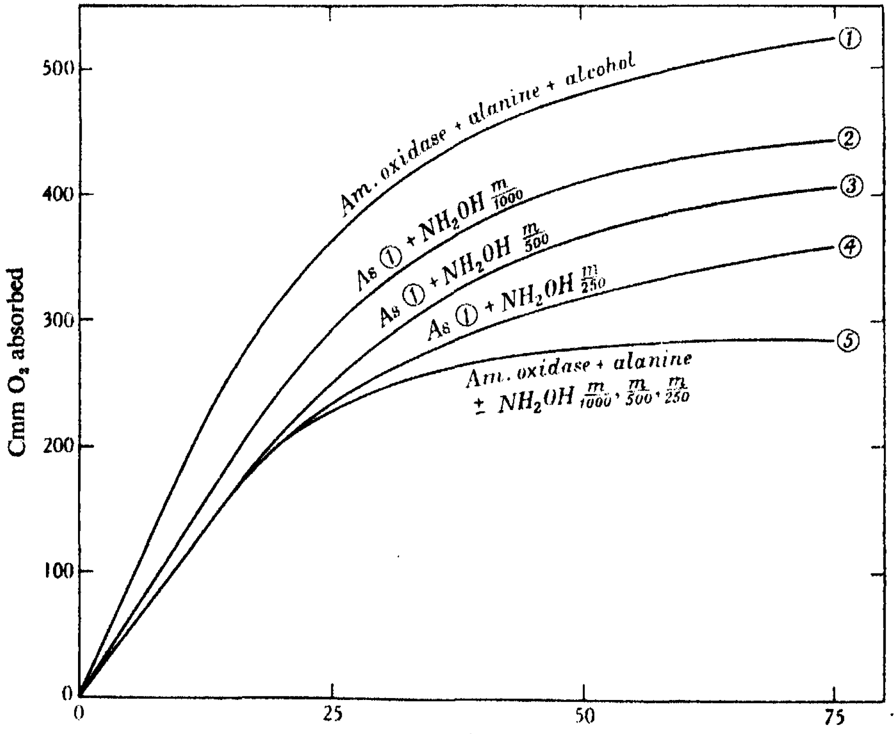


FIG. 8—Inhibition by hydroxylamine of the coupled oxidation of alcohol by amino acid oxidase.

We have observed, however, that ferric tannate, like haematin but unlike inorganic iron salts, strongly catalyses the decomposition of ethyl hydroperoxide into aldehyde. It is thus likely that in Stoecklin's experiments, the appearance of aldehyde was due to the action of ferric tannate on an organic peroxide slowly produced in the reaction mixture during 18–20 hours.

We can say, in conclusion, that the coupled oxidation of alcohol requires, in addition to hydrogen peroxide formed in the primary reaction, the co-operation of a second factor, which may be either a specific dehydrogenase activating alcohol, or catalase, or an organic compound of iron present in all catalase preparations so far examined.

The supposition that it may be alcohol dehydrogenase is supported by the specificity of the reaction and by the fact that this dehydrogenase is widely distributed and may be present in all our preparations except that of xanthine oxidase. This supposition is, however, opposed by the marked differences of the properties separating this factor from all known systems activating alcohol, namely: (1) it does not seem to reduce methylene blue; (2) it is highly resistant to acetone, chloroform, ether, and drying; (3) it can remain active for months in solution; and (4) it can be poisoned by KCN, $NaN_3$, and $NH_2OH$.

The suggestion that it is catalase itself is strongly supported by the extraordinary parallelism between this reaction and the presence of catalase, by its presence in highly purified preparations of catalase, by great similarity in resistance to some of the organic solvents, and by the effect of specific inhibitors. According to this supposition, the physiological function of catalase would be mainly peroxidatic and only in exceptional cases a catalatic one. This would partly explain the peculiar mutual exclusion of the two enzymes, catalase and peroxidase, namely, that the cells rich in catalase are generally very poor in, or free from, peroxidase and vice versa. This supposition does not, however, help us to understand the specificity of this coupled oxidation of alcohol, especially if the reaction consists mainly in some activation of nascent hydrogen peroxide.

The supposition that it may be an undetermined iron compound is supported by the fact that iron is present in all our enzyme preparations and that some iron compounds are supposed to catalyse the oxidation of alcohol by hydrogen peroxide; but we have not yet succeeded in separating such a compound from catalase itself.

## VII—SUMMARY

The addition of alcohol to the mixtures of uricase and uric acid or to amino acid oxidase and amino acids, doubles the oxygen uptake by these primary oxidation systems.

The addition of alcohol to xanthine oxidase and hypoxanthine or to xanthine oxidase and aldehyde has no effect on the oxygen uptake of these systems.

If, however, in addition to alcohol, a little purified catalase preparation is added to the xanthine oxidase system, the oxygen uptake becomes more than double that of the primary oxidation system.

In all these cases alcohol undergoes a secondary or coupled oxidation to aldehyde by $H_2O_2$ formed in the primary oxidation reaction.

While catalase preparation mixed with ordinary hydrogen peroxide has no effect on alcohol, the same preparation when added to other peroxides such as barium peroxide, cerium peroxide, ethyl hydroperoxide, and peroxide formed in the primary oxidation reaction, catalyses the oxidation of alcohol to aldehyde.

The only property which seems to be common to all these peroxides is the gradual liberation by them of hydrogen peroxide in what may be considered as a nascent state.

The coupled oxidation of alcohol to aldehyde requires, therefore, the co-operation of two factors: (1) a peroxide in nascent state which is being constantly formed in the primary oxidation reactions catalysed by uricase, amino acid oxidase, and xanthine oxidase; and (2) a factor found in uricase, amino acid oxidase, and catalase preparations but not in xanthine oxidase preparation.

As to the nature of this second factor, the study of the secondary oxidation reaction points to three possibilities which have already been discussed in detail, namely, that it may be: (1) an enzyme activating alcohol in a special way so that it can react only with nascent hydrogen peroxide; (2) the catalase itself which may react with this peroxide in a manner different from its reaction with ordinary $H_2O_2$, and activating it so that it can oxidize alcohol; (3) a special compound of iron and protein present in preparations of uricase, amino acid oxidase, and catalase but not in that of xanthine oxidase.

In the present state of our knowledge, most of the evidence appears to be in favour of the second supposition.

In a system composed of xanthine oxidase + aldehyde + alcohol + catalase preparation, aldehyde undergoes a primary oxidation to the corresponding acid with the formation of $H_2O_2$ which promotes the secondary oxidation of alcohol to aldehyde, and this in its turn is oxidized to acid. In other words, the secondary oxidation continually supplies the substrate for the primary oxidation reaction and the oxidation proceeds far beyond doubling the oxygen uptake of the primary reaction. This type of oxidation system is described here as cyclic oxidation.

## REFERENCES

Battelli, F., and Stern, L. (1909). 'C.R. Soc. Biol. Paris,' vol. **67**, p. 419.
—— (1910). 'Biochem. Z.,' vol. **28**, p. 145.
Euler, H. v., and Adler, E. (1934). 'Z. physiol. Chem.,' vol. **226**, p. 195.
Harrison, D., and Thurlow, S. (1926). 'Biochem. J.,' vol. **10**, p. 217.
Lehmann, J. (1934). 'Biochem. Z.,' vol. **272**, p. 144.
Müller, B. (1931). 'Biochem. Z.,' vol. **262**, p. 239.
—— (1934). *Ibid.*, vol. **268**, p. 152.
Stoecklin, E. de (1909). 'C.R. Acad. Sci. Paris,' vol. **148**, p. 424.
Stoecklin, E. de, and Vulquin, E. (1909). 'C.R. Acad. Sci. Paris.,' vol. **148**, p. 1404.
Thurlow, S. (1925). 'Biochem. J.,' vol. **19**, p. 175.
Tsuchihashi, M. (1930). 'Biochem. Z.,' vol. **140**, p. 63.
Wieland, H., and Chrometzka, F. (1930). 'Ber. deuts. chem. Ges.,' vol. **63**, p. 1028.
Zeile (1930). 'Z. physiol. Chem.,' vol. **195**, p. 29.
—— and Hellström (1930). 'Z. physiol. Chem.,' vol. **192**, p. 171.

---

577 · 158 · 431

# The Action of Cyanide and Other Respiratory Inhibitors on Xanthine Oxidase

By M. DIXON, Sc.D., and D. KEILIN, F.R.S.

(From the Molteno Institute, University of Cambridge)

(*Received October* 21, 1935)

## I—INTRODUCTION

It was stated by Dixon and Thurlow (1925) that xanthine oxidase is not inhibited by cyanide. They found that concentrations of cyanide up to M/100 did not produce any inhibition of either the uptake of oxygen or the reduction of methylene blue by hypoxanthine in presence of the enzyme. Some inhibition was, however, observed with higher concentrations of cyanide.

During the course of some other work on xanthine oxidase, Dixon and Lemberg, using the methylene blue technique, observed in 1931 (unpublished) that even small concentrations of cyanide completely inactivated the oxidase, provided that it was incubated with the cyanide for some time at 37° before testing. Similar results were later obtained by

Keilin (unpublished) for $O_2$ uptake. A reinvestigation of the action of cyanide on the xanthine oxidase was therefore desirable, and the results obtained are described in the present paper. It has been found that the action of cyanide on this oxidase shows a number of peculiar features which distinguish it from the other cases of cyanide inhibition hitherto known.

## II—METHODS AND MATERIALS

Both the uptake of oxygen and the reduction of methylene blue by the system were studied. The oxygen uptake was measured in Barcroft manometers in the usual way. As $CO_2$ is not produced in the reaction, there was no need for alkali in the absorption tubes, and the danger of removal of HCN by absorption during the experiment was consequently avoided. In the methylene blue technique the improvements described by Green and Dixon (1934) were adopted throughout.

As the reduction of methylene blue proceeds approximately linearly, for reasons discussed later, the reciprocal of the reduction-time is a measure of the activity. With oxygen, however, the uptake is not linear, except just at first, mainly owing to the progressive destruction of the oxidase by hydrogen peroxide formed during the oxidation of the hypoxanthine, and it is therefore necessary to take the *initial* velocity as a measure of the activity.

The activity with oxygen is expressed by the symbol $-Q_{O_2}$, this denoting as usual cmm $O_2$ absorbed per hour per mg dry weight of enzyme preparation. In order to bring the methylene blue results into line with this, we propose the symbol $-Q_{MB}$, denoting the number of cmm $O_2$ equivalent to the methylene blue reduced per hour per mg of enzyme preparation. This is easily obtained from the reduction time; for instance, at the beginning of fig. 1, 2 cc of 8% solution of oxidase preparation (with 0·1 cc M/68 hypoxanthine) reduced 1 cc 0·0005 M methylene blue in 1 min 15 sec. This amount of methylene blue is equivalent to 5·6 cmm $O_2$. Now 5·6 cmm in 75 sec is equivalent to 269 cmm per hour. This was due to 160 mg of enzyme preparation, so that 1 mg of enzyme would give 1·68 cmm per hour, which is the $-Q_{MB}$ value. The negative sign denotes that the oxygen was absorbed and not evolved, in accordance with current practice in dealing with cell-respiration systems. For milk, as the dry weights were unknown, the results are expressed per cmm of milk, the symbol $q_{MB}$ being used.

Preparations of the oxidase were made from milk by three different methods: the caseinogen preparation of Dixon and Thurlow (1924, *a*), the whey preparation of Dixon and Kodama (1926), and the preparation of Dixon and Lemberg (*see* Green and Dixon, 1934). Solutions of these were made up in phosphate buffer to give a $p_H$ of approximately 7·4. The cyanide solutions were always neutralized before use.

## III—INACTIVATION BY CYANIDE

### 1—*Course of Inactivation*

If cyanide is added to a solution of xanthine oxidase and it is allowed to stand, a progressive inactivation of the enzyme occurs, and proceeds until the enzyme is completely inactive. The rate of inactivation depends on the temperature and on the cyanide concentration; with M/400 cyanide 98% of the enzyme is inactivated in 45 min at 38°, but only 40% is inactivated at 20° during the same period; the rate increases rapidly with increase of cyanide concentration. These facts are shown by the following experiments.

Solutions of xanthine oxidase were made up in phosphate buffer and warmed to 38°. The correct amount of freshly neutralized M/25 cyanide to give the required concentration was then added with rapid mixing and the solution incubated in the thermostat. Samples were withdrawn at intervals and their activity towards hypoxanthine tested by measuring either the rate of reduction of methylene blue anaerobically or the rate of uptake of oxygen.

Fig. 1 shows the inactivation of an 8% solution of the caseinogen preparation at 38° by M/500 HCN, as followed by the methylene blue technique using hypoxanthine as substrate. Parallel measurements by the oxygen uptake technique gave the same results, as shown in the lower curve of fig. 3. The fact that the same results are obtained with methylene blue and with molecular oxygen is of importance, as it shows that the cyanide is not (as in other cases of cyanide inhibition) acting upon some oxygen-activating catalyst present in the system but upon the dehydrogenase itself. It is the activation of the hypoxanthine which is inhibited by the cyanide, and the effect consequently gives no evidence of oxygen-activation in the xanthine oxidase system. This is the first known case of an enzyme capable of catalysing anaerobic oxidations being inhibited by cyanide: the other enzymes of this type, so far as they have been tested, appear to be completely resistant to cyanide.

### 2—*Effect of Temperature*

Fig. 3 shows the effect of temperature on the rate of inactivation by cyanide. For the upper curve the enzyme and cyanide were kept at

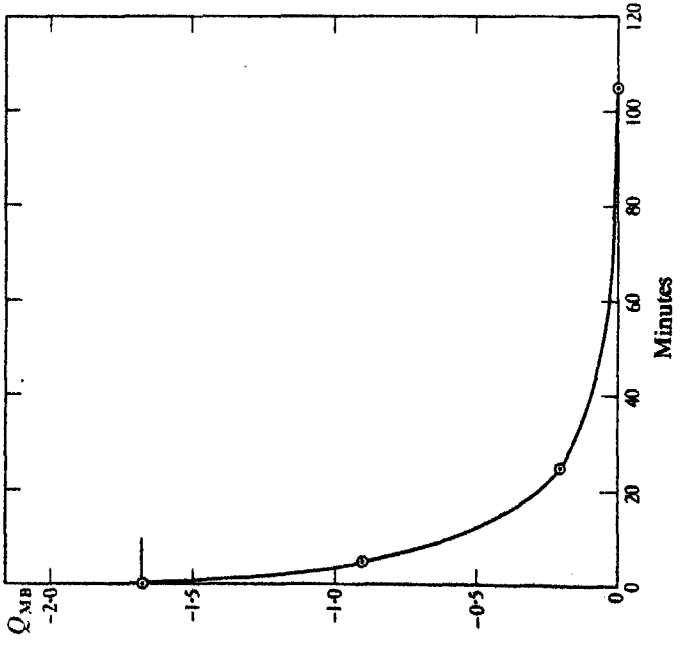

$Q_{MB}$
-2·0
-1·5
-1·0
-0·5
0
0
20
40
60
80
100
120
Minutes

FIG. 1.

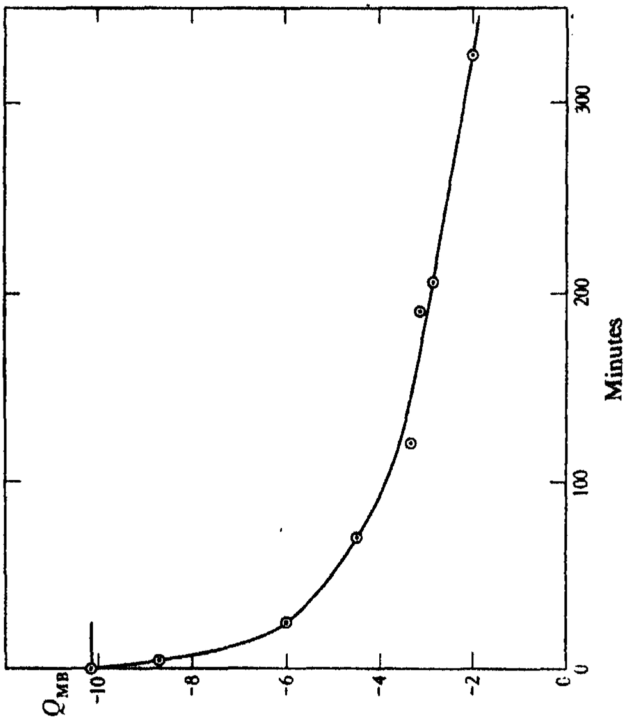

$Q_{MB}$
-10
-8
-6
-4
-2
0
0
100
200
300
Minutes

FIG. 2.

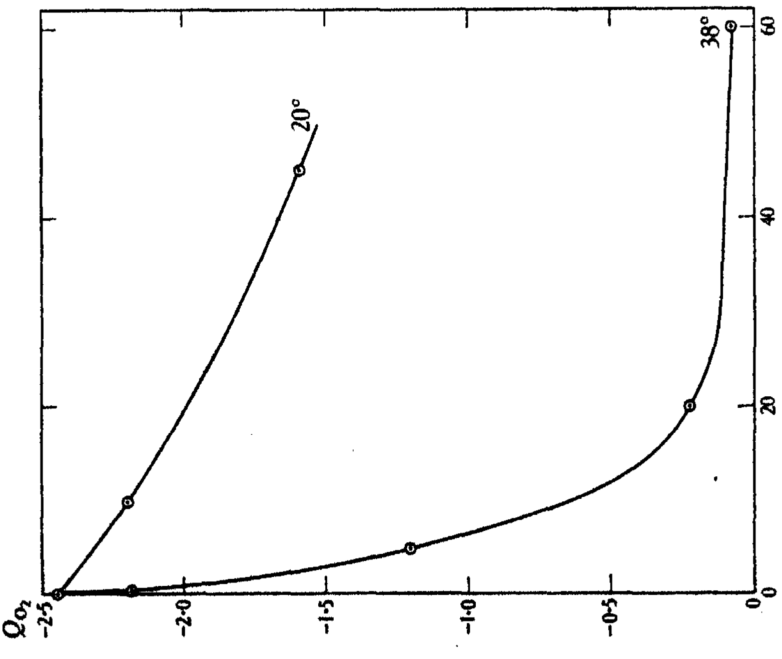


FIG. 3.

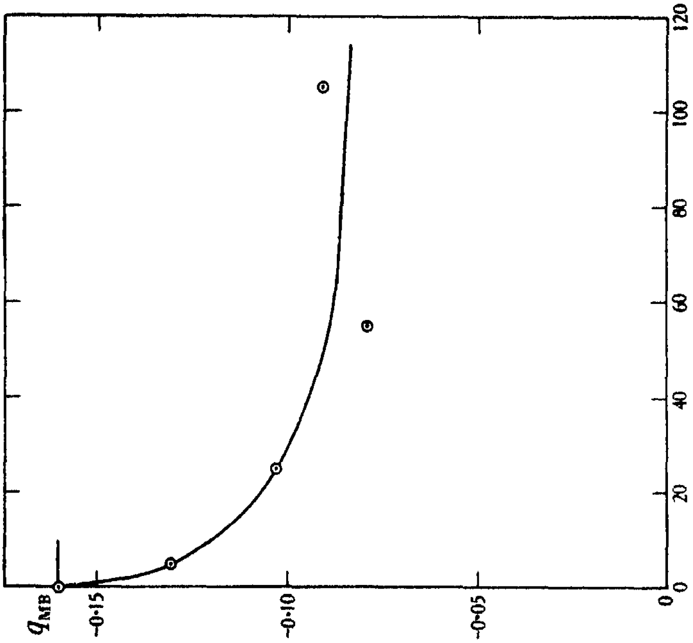


FIG. 4.

FIGS. 1–4—Inactivation of xanthine oxidase by cyanide. Fig. 1, caseinogen, M/500 HCN; fig. 2, whey, M/10,000 HCN; fig. 3, caseinogen, M/500 HCN; fig. 4, milk, M/500 HCN.

20°, the measurements of activity being carried out at 38° as usual. *It will be seen that at room temperature the oxidase is only very slowly inactivated by cyanide. The relative slowness with which cyanide acts* on the xanthine oxidase is in marked contrast with all other known cases of inhibition of oxidases, catalytic iron compounds, etc., as in these the action of the cyanide is instantaneous.

### 3—*Extent of Inactivation*

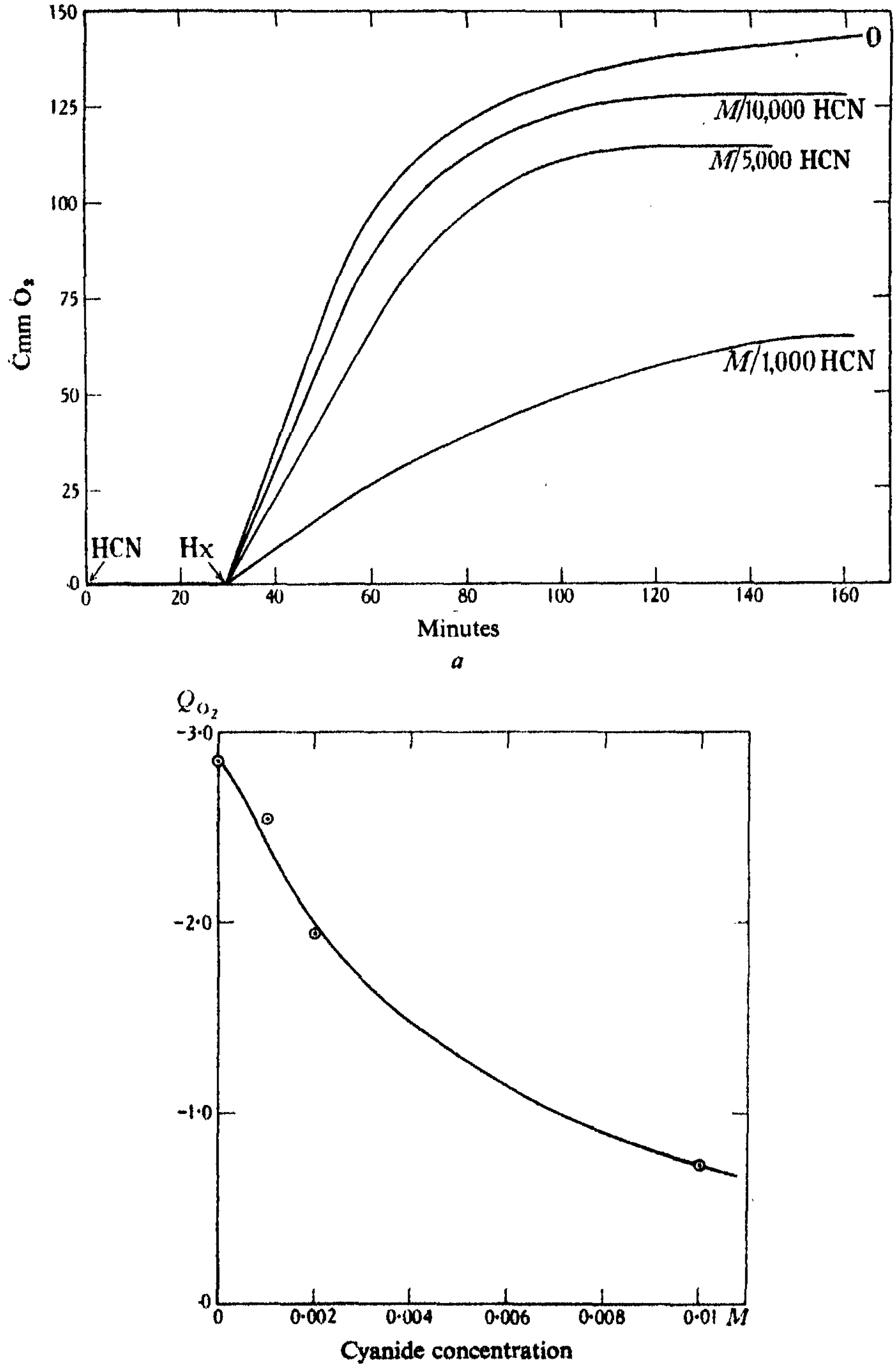
It will be noticed that, provided the cyanide is allowed to act for a sufficient time, the inactivation is complete. With more dilute cyanide the rate of inactivation is less, but even with very small concentrations the inactivation is ultimately complete, although it may be only after many hours. Fig. 2 shows the effect of M/10,000 HCN upon a 4% solution of the whey preparation, which is rather more rapidly acted upon by cyanide than the caseinogen preparation. A comparable degree of inhibition by cyanide of this dilution is rarely met with among oxidases.

Ultimately complete inactivation is invariably found with preparations of the oxidase, and usually with milk, but on rare occasions a sample of milk is obtained in which the inactivation remains incomplete, and one such case is shown in fig. 4. This effect, which is quite exceptional, has not yet been explained, but it appears that a certain fraction of the oxidase in these cases is in some way protected against the action of the cyanide.

### 4—*Effect of HCN Concentration*

The relation between the cyanide concentration and the rate of inactivation of the oxidase is shown in fig. 5. In this experiment 70 mg of whey preparation dissolved in 3 cc of buffer solution containing the stated concentrations of cyanide were placed in each of four Barcroft manometers, and after shaking for 30 min at 38° 1 mg of hypoxanthine in solution was added to each from a "dangling-tube." The actual oxygen uptake curves are shown in fig. 5*a*, and the activities remaining after the 30 min exposure to cyanide are plotted against the cyanide concentration in fig. 5*b*.

In fig. 5*a* it will be noticed that not only is the rate of oxygen uptake decreased by the cyanide but also the total amount taken up. In the lower curves it is found that the oxidase is completely destroyed at the end of the experiment. It will be clear from results given later that this is not due to a further action of the cyanide after the addition of the hypoxanthine, but that it is due mainly to destruction by hydrogen



*a*

*b*

Fig. 5—Effect of cyanide concentration.

peroxide. Precisely similar curves are, in fact, obtained in the absence of cyanide by varying the amount of oxidase initially present, as shown by Dixon (1925, p. 508).

### 5—*Necessity of Incubation*

In order to produce any effect, the cyanide must act upon the oxidase for a definite time; in other words, the mere presence of the cyanide, without previous incubation with the enzyme, has no effect upon the reaction. Thus the initial points of the inactivation curves given above are the same as the control values without cyanide. The following experiment shows this fact clearly.

2 cc of 8% xanthine oxidase solution (caseinogen preparation) + 1 cc 0·0005 M methylene blue were measured into each of five Thunberg tubes, and 0·15 cc M/68 hypoxanthine was placed in each of the hollow stoppers. To tubes 1*a* and 1*b*, which were duplicate controls, 0·25 cc water were also added, while 0·25 cc M/30 cyanide were added to tubes 3 and 4 and to the hollow stopper of tube 2. All the tubes were evacuated, filled with $O_2$-free nitrogen, and again evacuated as usual before being placed in the thermostat at 38°. After gentle shaking for a short time, to bring the contents of the tubes to the temperature of the thermostat, the tubes were inverted so as to mix the solutions (except in the case of tube 4, the contents of which were mixed 20 min later). Thus in tube 2 the cyanide was added simultaneously with the hypoxanthine and hence any previous action of cyanide on the oxidase was avoided, while in tube 3 the cyanide was in contact with the enzyme for about 3 min at room temperature (during evacuation) and ½ min at 38°, which is roughly equivalent to 1 min at 38°. The results are given in Table I.

TABLE I

| Tube No. | Time of action of cyanide | Reduction time |
|---|---|---|
| 1*a* | Control | 32 sec |
| 1*b* | „ | 33 „ |
| 2 | 0 | 35 „ |
| 3 | 1 min | 45 „ |
| 4 | 20 „ | 25 min + |

It will be seen that when the cyanide is added simultaneously with the hypoxanthine it has no effect.

### 6—*Irreversibility of Inactivation*

The fact that xanthine oxidase is ultimately inactivated completely, even by cyanide which is so dilute that it acts only slowly, is in marked contrast to the results obtained in other cases of cyanide inhibition. In these cases there is a definite equilibrium between the cyanide and the catalyst, and the degree of inhibition depends on the cyanide concentration, any given concentration being associated with a definite percentage inhibition which is set up at once and does not change with time. In these cases the catalyst recovers its activity completely on removal of the free cyanide by physical or chemical means. The results described above, on the other hand, suggest that the inactivation of xanthine oxidase by cyanide is irreversible, and the following experiments show that this is, in fact, so.

An 8% solution of the caseinogen preparation was made up and divided into two portions. One portion served as a control, while to the other cyanide was added to give a concentration of M/400. They were then incubated at 38° until 98% of the enzyme had been inactivated by the cyanide. The oxidase was then precipitated in both cases by adding an equal volume of saturated ammonium sulphate solution, filtered off and washed with half-saturated ammonium sulphate. The precipitates were finely dispersed in half-saturated ammonium sulphate and again filtered off. They were then redissolved in dilute phosphate buffer, reprecipitated with ammonium sulphate, filtered off, and dried as usual. By this means every trace of cyanide was removed. 8% solutions were then made up, and the activity tested. It was found that the control portion had not changed in activity and the cyanide-treated portion still showed a relative inactivation of 98%.

In other experiments the free cyanide was removed by the addition of excess of methaemoglobin, which readily combines with it. Other systems (*e.g.*, uricase (Keilin and Hartree, 1935)) when poisoned with cyanide recover their full activity on addition of methaemoglobin, but with xanthine oxidase it was found that methaemoglobin had no restoring action. The methaemoglobin was prepared from three times recrystallized horse haemoglobin by the action of ferricyanide followed by thorough electrodialysis. Fig. 6 shows a number of oxygen uptake curves, in each case of 150 mg caseinogen preparation + 1 mg hypoxanthine in a total volume of 3 cc. Curve 1 shows the uptake by the oxidase and hypoxanthine alone. Curve 5 was given by the oxidase after incubating for 2 hours with M/500 HCN, and curve 6 is the same as curve 5, but with the addition, after the incubation, of 0·7 cc 15% methaemoglobin, followed 15 min later by the hypoxanthine. For curve 3 the oxidase was previously incubated for 30 min with the cyanide, and curve 4 is the same with methaemoglobin added after the incubation. Curve 2 shows the effect of the methaemoglobin on the oxidase without cyanide.

It will be seen that curves 5 and 6 are coincident, showing that when the oxidase is inactivated by cyanide the methaemoglobin has no restoring action. Methaemoglobin has, however, another action. When added to the intact oxidase it increases the total oxygen uptake up to almost twice the theoretical amount. This effect, already observed by Harrison and Thurlow (1926), is due to a peroxidase-like action of methaemoglobin which enables the hydrogen peroxide formed during the oxidation of hypoxanthine to be used for the oxidation of other substances present in the preparation. The extra oxygen taken up is due to the oxidation of these substances. The hydrogen peroxide, being thus used, is removed from the sphere of action and no longer produces the progressive destruction of the oxidase which is the main cause of the falling off of the velocity in curve 3. Thus the methaemoglobin protects the enzyme from destruction by peroxide, and, as seen in curve 4, the whole reaction proceeds with approximately the initial (inhibited) velocity. The difference between curves 3 and 4 is therefore not to be interpreted as showing a reversal of the cyanide effect by methaemoglobin. It will be noticed that initially the velocity is the same in both curves.

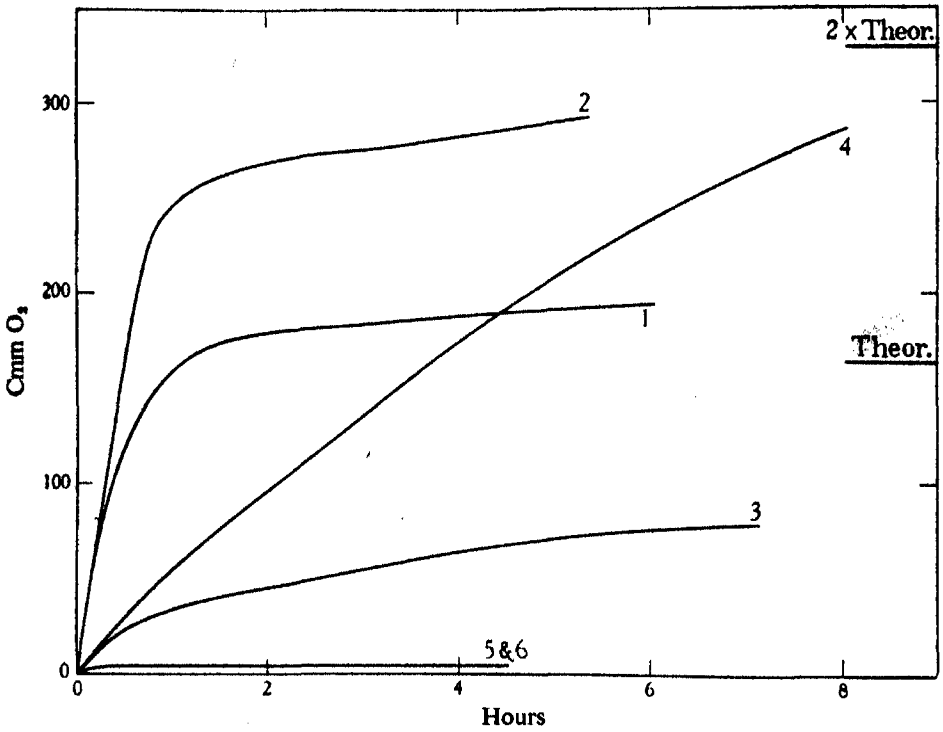


FIG. 6—Effect of adding methaemoglobin.

It may be concluded from the above results that the action of cyanide on xanthine oxidase is irreversible.

## IV—PROTECTION BY PURINES

### 1—*Apparent Discrepancy with Previous Work*

The results given above apparently contradict the observations of Dixon and Thurlow (1925), who could detect no inhibition by cyanide and found the oxygen uptake curves with and without cyanide to be coincident during the whole course of the reaction. For in their case the reaction extended over a period of about 1½ hours, during the whole of which time the enzyme was in contact with cyanide at 38°, so that even without previous incubation a marked inhibition should have developed during the reaction (*cf.* fig. 3 above). Even with M/300 HCN, however, no trace of inhibition was found.

We have confirmed the experiments of Dixon and Thurlow completely, and the elucidation of the cause of the apparent discrepancy has brought to light an interesting phenomenon.

The results obtained depend upon the order and time intervals of adding the reagents. If the oxidase and cyanide are added to the Barcroft flask, and the hypoxanthine is added from a " dangling tube " after the equilibration period, a marked inhibition is obtained, which is greater the greater the time interval between the addition of the cyanide and the hypoxanthine. In this case the cyanide has time to act before the substrate is added. If, however, the hypoxanthine is added before the cyanide no inhibition is produced. In other words, the hypoxanthine protects the oxidase against the action of the cyanide. Now in the experiments of Dixon and Thurlow the cyanide was added to the oxidase at room temperature, followed after about 1 min by the hypoxanthine, after which the manometer was transferred to the thermostat at 38°. As the action of cyanide is slow at room temperature there was thus no time for inactivation to take place before the enzyme was protected by the addition of the hypoxanthine, and consequently no effect was observed. These facts are illustrated by the following experiments.

### 2—*Protection by Hypoxanthine*

Fig. 7 shows that the addition of cyanide while the oxidation of hypoxanthine is actually in progress has no inhibitory effect. Each manometer contained 200 mg of the caseinogen preparation + 1 mg hypoxanthine in a total volume of 3 cc. In one manometer 0·2 cc M/28 HCN (giving a final concentration of M/450) was added from a "dangling tube" at the point shown by the arrow. Curve 2 shows the oxygen uptake in this manometer, curve 1 being the control without cyanide.

For the experiment shown in fig. 8, manometers with special flasks provided with side-bulbs were used. The cyanide (to give a final concentration of M/500) was placed in the side-bulbs and the hypoxanthine in "dangling tubes." Thus the hypoxanthine and cyanide could be added separately to the oxidase when desired. 80 mg caseinogen preparation and 1 mg hypoxanthine were used in each manometer. Curve 1 was given by the control without cyanide; when the hypoxanthine was added 10 min after the cyanide, curve 3 was obtained, and when the hypoxanthine was added immediately before the cyanide, curve 2 resulted.

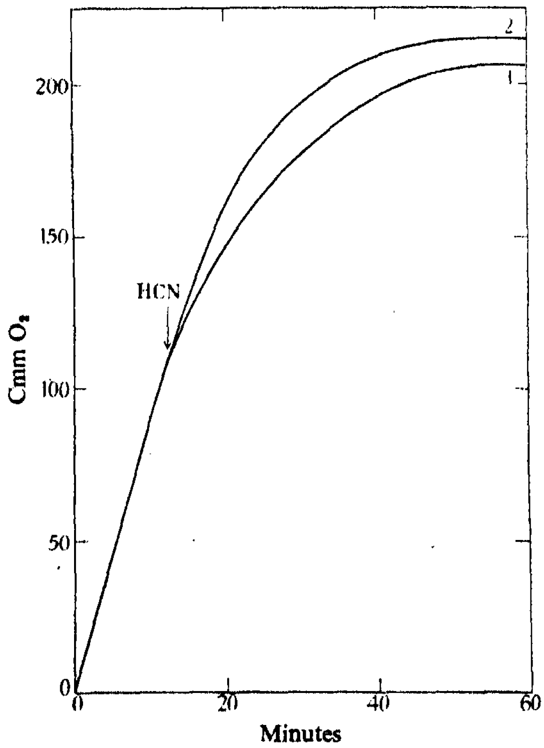


FIG. 7—Addition of HCN during oxidation.

In both these experiments it is clear that the hypoxanthine protects the oxidase from the action of the cyanide, and this protective action explains the results obtained by Dixon and Thurlow. The most natural interpretation of the effect is that the cyanide and hypoxanthine both combine with the same active grouping of the enzyme, so that when the enzyme is combined with hypoxanthine it is unable to combine with cyanide and so become inactivated. This interpretation involves the assumption that the affinity of the enzyme for hypoxanthine is much greater than its affinity for cyanide, and, in fact, it is known from the work of Dixon and Thurlow (1924, *b*) that the affinity of xanthine oxidase for hypoxanthine is exceptionally high. Certain facts to be given below, however, show that this simple interpretation is inadequate.

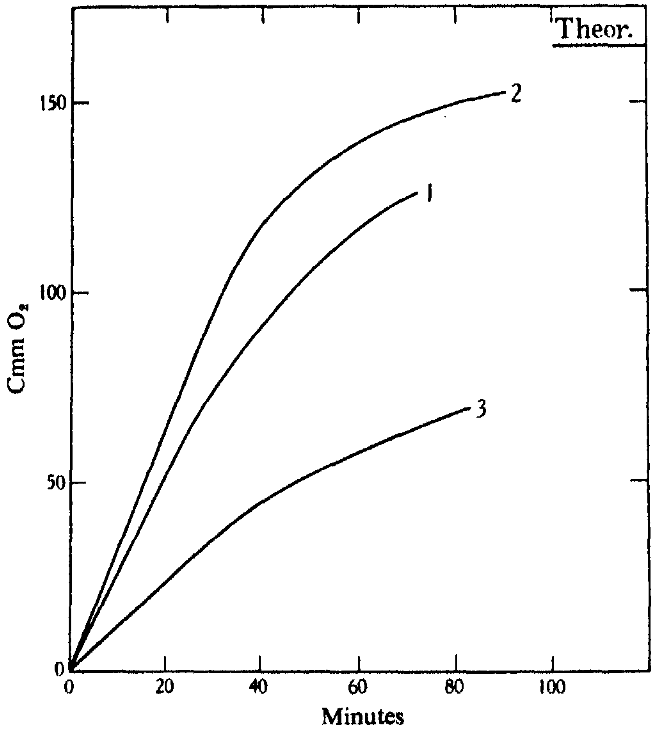


FIG. 8—Effect of order of addition of reagents.

It is clear that once the hypoxanthine has been added and its oxidation commenced the cyanide has no further effect. This is also the case when methylene blue is substituted for oxygen. In the following experiment the reduction time was purposely prolonged to about eight times its normal value by using eight times as much methylene blue as usual. In two Thunberg tubes were placed 2 cc 8% caseinogen preparation + 2 cc 0·002 M methylene blue, and 0·2 cc M/68 hypoxanthine in each of the hollow stoppers. To one of the stoppers 0·25 cc M/30 cyanide was also added, and 0·25 cc water to the other. The contents were mixed as usual after evacuating and placing in the thermostat; thus there was no previous incubation with cyanide. The reduction times were as follows:

| | |
|---|---|
| Without HCN ………… | 17 min 10 sec |
| With HCN ………….. | 21 min 45 sec |

Now had the action of the cyanide been proceeding during the reaction the reduction time would have been very greatly prolonged, for the greater part of the enzyme would have been destroyed in this time. Actually, however, the cyanide has had only a slight effect, showing that the inactivation had been practically prevented. The reduction proceeded at a practically constant rate in both cases.

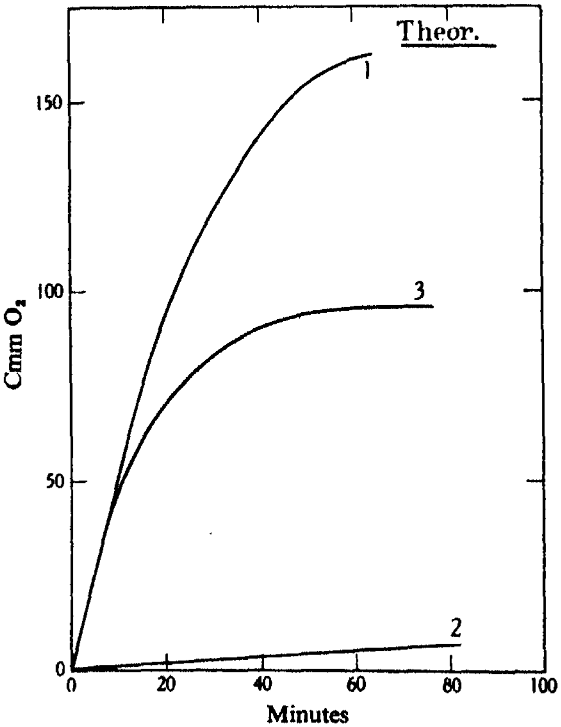


Fig. 9—Protection against high HCN concentration by hypoxanthine.

It was mentioned above that Dixon and Thurlow observed some inhibition with high concentrations of cyanide (greater than M/100). This is simply due to the fact that the rate of inactivation increases with the cyanide concentration, and with such high concentrations the action may be appreciable even at room temperature during the short interval before the addition of hypoxanthine in their experiments. If the hypoxanthine is added before the cyanide it can protect the oxidase against even higher concentrations, although in this case not for an indefinite time, as inhibition develops in the later stages of the reaction. In fig. 9, curve 1 shows the oxygen uptake of 120 mg caseinogen preparation + 1 mg hypoxanthine without cyanide; curve 2 is the same except for the presence of M/50 HCN (final concentration) added 15 min before the hypoxanthine, and curve 3 is the same as curve 2 except that the hypoxanthine was added just before the cyanide. It will be seen that even with M/50 cyanide the hypoxanthine gives complete protection during about one-third of the reaction.

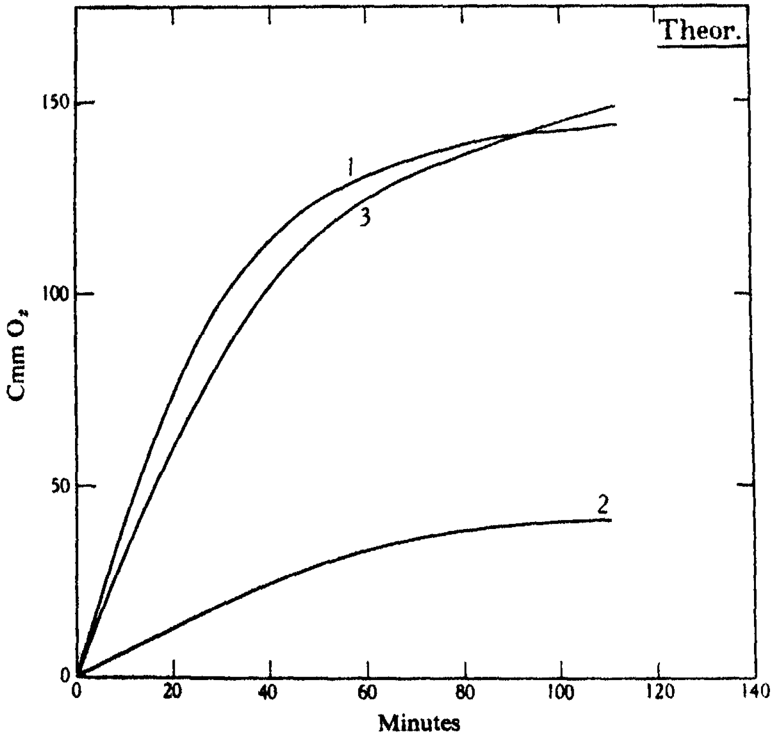


Fig. 10—Protection by uric acid.

### 3—*Protection by Uric Acid, etc.*

Xanthine oxidase has a high affinity for other purines (*e.g.*, uric acid, adenine, guanine, but not purines such as caffeine) in addition to hypoxanthine, and if the above explanation is correct these purines should also protect the enzyme against the action of cyanide. This is actually found to be so; uric acid, adenine, and guanine all protect the oxidase, but caffeine has no effect whatever. Fig. 10 shows the protective action of uric acid. Curve 1 is the control, showing the oxygen uptake of 120 mg caseinogen preparation + 1 mg hypoxanthine without cyanide. For curve 2 the enzyme was incubated for 20 min with cyanide (M/450) before the hypoxanthine was added, while curve 3 is the same as curve 2 except that 3 mg of uric acid was added before the cyanide. Thus in curve 3 uric acid was present during the period of incubation with cyanide, and, as the curves show, the oxidase was practically completely protected by it. Exactly similar results were obtained using guanine instead of uric acid. Neither uric acid nor guanine affected the rate of oxygen uptake in the absence of cyanide.

The degree of protection by uric acid depends upon its concentration, as shown in fig. 11. In six Barcroft manometers were placed xanthine oxidase solutions (80 mg caseinogen preparation per manometer) containing varying concentrations of uric acid. Cyanide in a concentration of M/500 was added to each, and after 10 min at 38° 1 mg hypoxanthine was introduced. The resulting activities (initial uptake rates) are plotted against the concentration of uric acid in fig. 11. The upper horizontal line shows the rate obtained in a seventh manometer without cyanide, and thus corresponds to complete protection, while the lower line shows the rate with cyanide but with no uric acid, and thus corresponds to zero protection. It will be seen that an appreciable degree of protection is given by even M/10,000 uric acid. An eighth manometer with M/500 caffeine instead of uric acid showed a rate coinciding with the line of zero protection.

Similar results were obtained by the methylene blue technique, although in this case a complication is introduced by the fact that the reduction of methylene blue by xanthine oxidase is partially inhibited by uric acid, as shown by Dixon and Thurlow (1924, *b*). It is therefore necessary to arrange the experiment so that while varying amounts of uric acid are present in the different tubes during the incubation with cyanide the same amount is always present during the subsequent testing of the activity with methylene blue and hypoxanthine. This was achieved by placing the required amounts of uric acid in the Thunberg tubes together with the oxidase and cyanide, and placing additional amounts of uric acid along with the hypoxanthine and methylene blue in the hollow stoppers so as to make up a constant total amount of uric acid in all cases. In the experiment shown in fig. 12 the tubes contained 2 cc 8% caseinogen preparation containing M/400 cyanide, 1 cc 0·0005 M methylene blue, 0·2 cc M/68 hypoxanthine, and a total amount of 0·2 cc uric acid (3 mg per cc). They were evacuated in the usual way and incubated 14 min at 38° before mixing the contents of the tubes and stoppers together. The upper horizontal line represents the activity without either uric acid or cyanide, the next line shows that obtained with uric acid but without cyanide, representing complete protection, and the lowest line that obtained with cyanide but with the whole of the uric acid placed in the stopper, representing zero protection. 0·13 cc of uric acid gave a concentration of 0·001 M during the incubation.

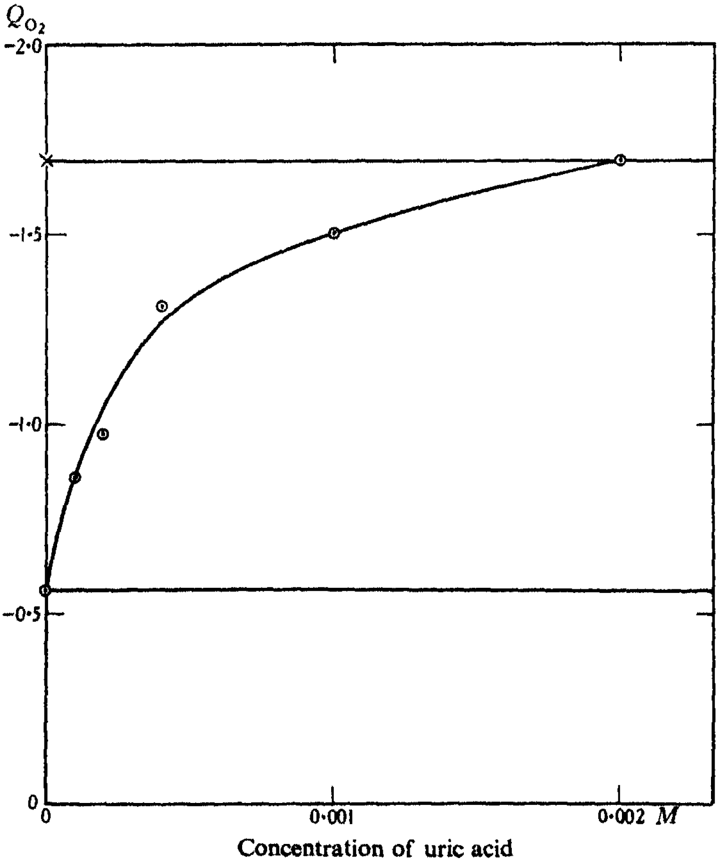


FIG. 11—Effect of uric acid concentration aerobically.

The above results leave it undecided whether the effect of uric acid is to decrease the final extent of the inactivation or merely to reduce the rate of action of the cyanide. If the combination of oxidase and uric acid is reversible the latter alternative would be expected. The point was decided by following the course of the inactivation in the presence and absence of uric acid, the amount chosen being such as gave only a partial protection in previous experiments. Two 20-cc portions of an 8%

solution of the caseinogen preparation were taken; to one was added 1 cc (3 mg) uric acid solution, and cyanide was then added to both in a concentration of M/400. They were both incubated at 38°, and 2 cc samples withdrawn at intervals and tested with 1 cc 0·0005 M methylene blue and 0·1 cc M/68 hypoxanthine. The results in the absence and presence of uric acid are shown in fig. 13, curves 1 and 2 respectively, and it is clear that the uric acid simply reduces the rate of action of the cyanide, the inactivation ultimately proceeding to completion in both cases.

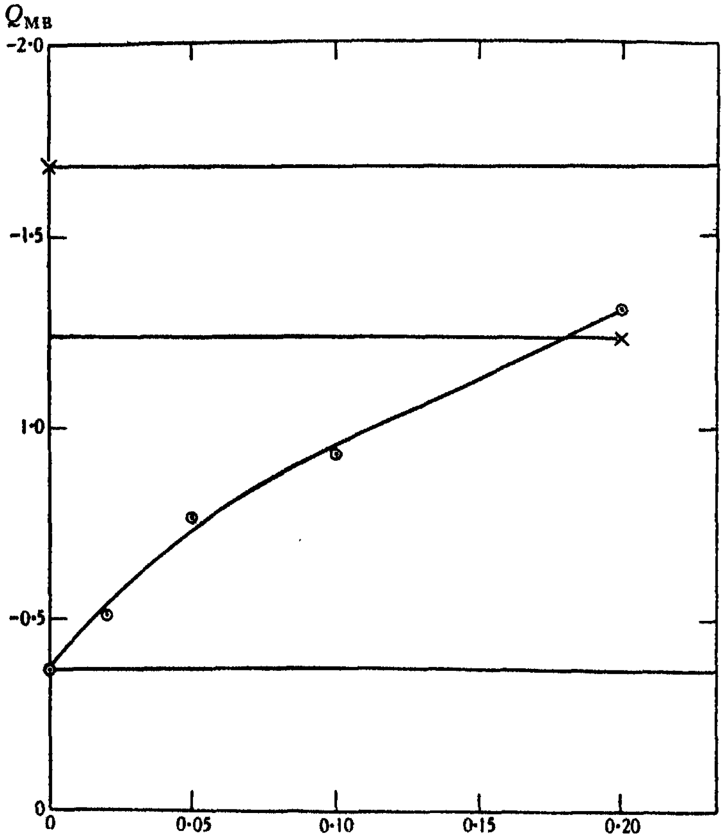


Fig. 12—Effect of uric acid concentration anaerobically.

## V—Anomalous Effects with Hypoxanthine

It has been shown above that hypoxanthine, when added initially, protects the oxidase against cyanide during its oxidation, either aerobically or anaerobically. Moreover, the presence of uric acid or other purines during preliminary incubation with cyanide likewise protects the enzyme. It appears, however, that the simple explanation suggested above (namely, that only the free oxidase, but not the oxidase-purine complex, is accessible to cyanide) is not the whole truth; for if this were all, the enzyme should be protected by hypoxanthine during preliminary anaerobic incubation with cyanide before adding the methylene blue. This has surprisingly been found not to be the case; on the contrary, under such conditions hypoxanthine may actually *increase* the effect of cyanide.

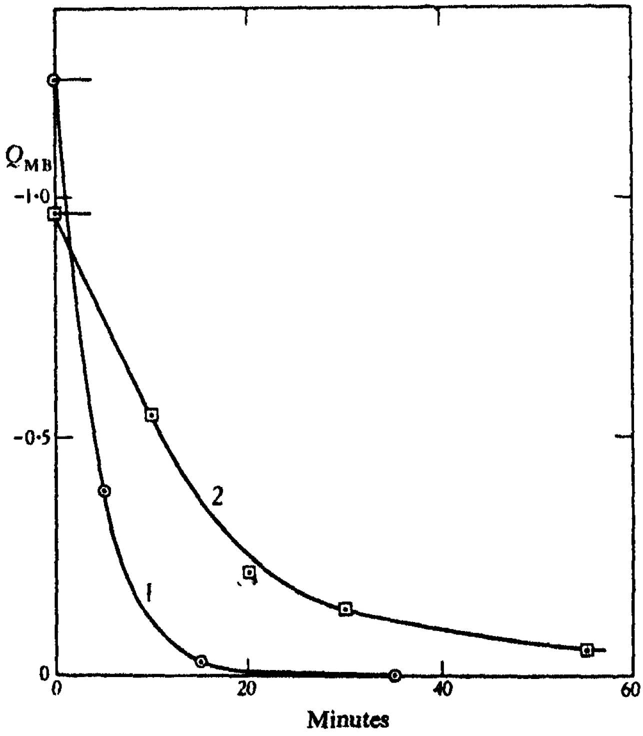


FIG. 13—Effect of uric acid on course of inactivation.

The contrasting behaviour of hypoxanthine and uric acid is clearly seen in the following experiment.

Five Thunberg tubes with hollow stoppers were filled as shown in the table. After freeing from oxygen they were incubated for 14 min at 38° before the contents of tubes and stoppers were mixed together. The resulting reduction times are shown in the last column of Table II.

The quantities used were: 2 cc 8% caseinogen preparation, 0·25 cc M/30 HCN, 1 cc 0·0005 M methylene blue (MB), 0·2 cc M/68 hypoxanthine (Hx), 0·2 cc uric acid (UA) (3 mg per cc).

A comparison of the first three tubes shows that the presence of hypoxanthine during the incubation with cyanide has considerably increased the inactivation, while the last two tubes show that the presence of uric acid during the same period has almost completely protected the oxidase (a slight inhibition by the uric acid itself must be allowed for in the last two tubes).

TABLE II

| Tube No. | Contents of tube | Contents of stopper | Reduction time min | Reduction time sec |
|---|---|---|---|---|
| 1 | Oxidase | MB + Hx | 1 | 15 |
| 2 | Oxidase + HCN + Hx | MB | 8 | 40 |
| 3 | Oxidase + HCN | MB + Hx | 2 | 50 |
| 4 | Oxidase + HCN + UA | MB + Hx | 1 | 35 |
| 5 | Oxidase + HCN | MB + Hx + UA | 5 | 5 |

The essential difference between this experiment and those given earlier, in which protection by hypoxanthine was obtained, is that here the oxidase is kept in contact with hypoxanthine and cyanide but is prevented from acting by the absence of the hydrogen acceptor (the methylene blue not being added until after the incubation), whereas in the earlier experiments the hydrogen acceptor was present and the oxidase reaction actually proceeding.

The behaviour of xanthine is in a sense intermediate between hypoxanthine and uric acid, for while it does not protect neither does it increase the cyanide inactivation. The experiment shown in Table III was carried out in the same way as the previous one.

TABLE III

| Tube No. | Contents of tube | Contents of stopper | Reduction time min | Reduction time sec |
|---|---|---|---|---|
| 1 | Oxidase | MB + X | 3 | 0 |
| 2 | Oxidase + HCN + X | MB | 37 | 30 |
| 3 | Oxidase + HCN | MB + X | 38 | 0 |
| 3 | Oxidase | MB + Hx | 1 | 30 |

(The velocity with xanthine is, as usual, half of that with hypoxanthine.)

Similar effects are obtained with oxygen as hydrogen acceptor instead of methylene blue. 1·5 cc of 8% caseinogen preparation of the oxidase were placed in special manometer vessels having side-bulbs and gas outlets. 1 mg of hypoxanthine in solution was placed in each side-bulb, and the appropriate quantity of cyanide solution to give a final concentration of M/450 was placed in dangling tubes in manometers 2, 3, and 4. To the oxidase solution in manometer 4 were also added 3 mg of uric acid. The manometers were placed in the water bath, and all traces of oxygen were expelled from the vessels by a current of pure nitrogen (freed from oxygen by passing over hot copper). In manometer 3 the hypoxanthine was now mixed with the oxidase solution, and the cyanide was then added in manometers 2, 3, and 4. After ½ hour at 38° the hypoxanthine was added in manometers 1, 2, and 4, the nitrogen was quickly displaced by air, and the oxygen uptake followed manometrically. Under these conditions, as shown in fig. 14, in which the numbers of the curves correspond with those of the manometers, the hypoxanthine, unlike the uric acid, had no protective action during the anaerobic incubation with cyanide.

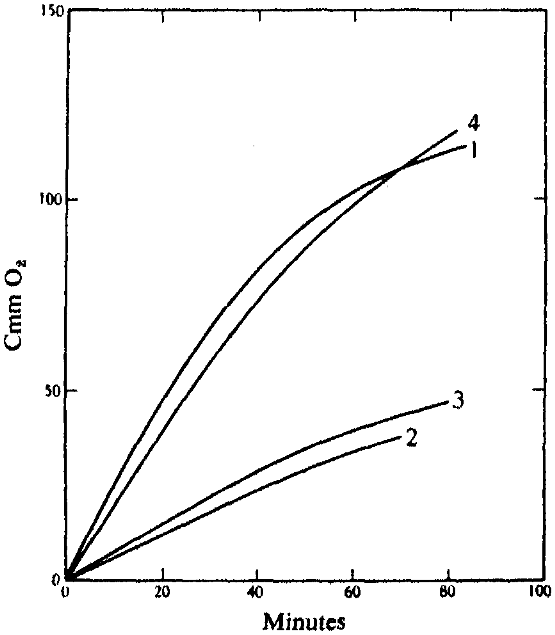


FIG. 14—Effect of previous anaerobic incubation.

The effects just described have been obtained many times with different caseinogen preparations using methylene blue, and some of the results are shown in the first part of Table IV. These experiments were carried out exactly as in the case of tubes 1 to 3 in Tables II and III, and the tubes are numbered to correspond. Milk behaves like the caseinogen preparation. With the whey preparation and the Dixon and Lemberg (NaCl) preparation, however, different results are obtained; in these cases hypoxanthine never increases the cyanide inactivation but always gives a partial protection, as shown in the second part of the table. It should be noted, however, that the whey preparation is more rapidly inactivated by cyanide than the others, and therefore the time of incubation was made shorter so as to obtain a comparable degree of inactivation.

TABLE IV

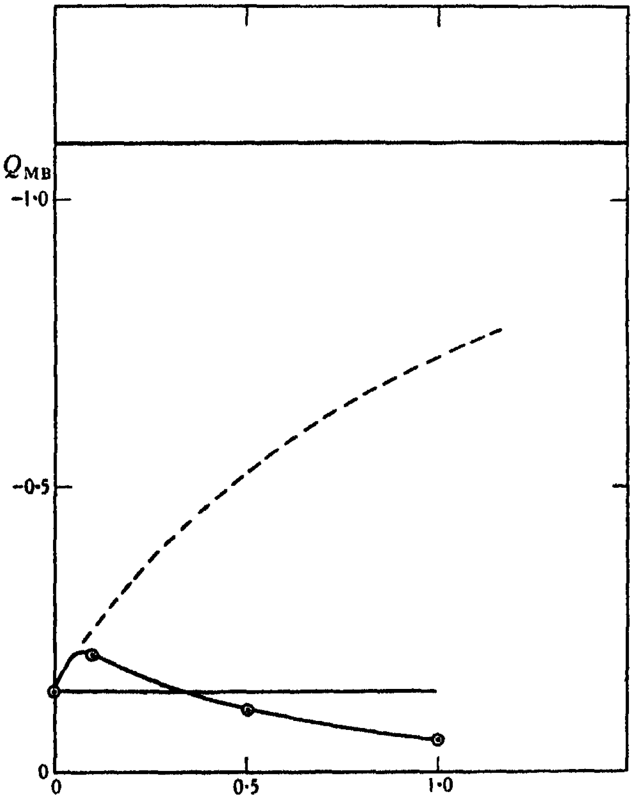
| Tube No. | Caseinogen preparations | | | | Milk |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | |
| | min sec | min sec | min sec | min sec | min sec |
| 1 | 1 15 | 1 20 | 1 40 | 1 32 | 1 10 |
| 2 | 8 40 | 8 58 | 11 50 | 10 5 | 2 30 |
| 3 | 2 50 | 3 10 | 4 52 | 5 25 | 1 40 |

| Tube No. | Whey preparations | | | Dixon and Lemberg preparation |
|---|---|---|---|---|
| | 1 | 2 | 3 | |
| | min sec | min sec | min sec | min sec |
| 1 | 0 20 | 1 52 | 0 17 | 0 16 |
| 2 | 0 39 | 2 42 | 0 38 | 0 43 |
| 3 | 1 13 | 4 40 | 0 45 | 1 7 |

8% solutions of the caseinogen preparations were used, and they and the milk were incubated with the cyanide for 14 min at 38°. The three whey preparations were made up in 8%, 1·6%, and 4% solution respectively, and were incubated for 5 min at 38°. The Dixon and Lemberg (NaCl) preparation was in 1% solution, and was incubated for 12 min at 38°.

It will be seen that in the first part of the table the greatest inactivation invariably occurs in tube 2, *i.e.*, that in which the hypoxanthine was present during the incubation with cyanide, while in the second part the greatest inactivation occurs in tube 3, in which the oxidase was incubated with cyanide alone. The reason for these differences in behaviour is not yet clear.

A more detailed study of the effect reveals that the results obtained depend upon the amount of hypoxanthine present during the incubation with cyanide. A very small amount produces some degree of protection in all cases, but as the amount is increased the protection becomes less. With the whey preparation the curve never reaches the line of zero protection, but with the caseinogen preparation it soon crosses this line and actually shows increased inactivation. Curves for the caseinogen and whey preparations respectively are shown in figs. 15 and 16. For fig. 15, 2 cc 8% caseinogen preparation + 0·25 cc M/30 HCN were placed in the tubes together with varying amounts of M/680 hypoxanthine (*i.e.*, one-tenth of the usual strength). In order to keep the amount of hypoxanthine present during the activity measurement uniform, the necessary amounts to make up a total of 1 cc were added to the hollow stoppers, together with the methylene blue (1 cc 0·0005 M). The tubes were incubated anaerobically for 14 min at 38° before mixing the contents. For fig. 16,



Cc M/680 hypoxanthine in tube during incubation

FIG. 15—Effect of hypoxanthine concentration with caseinogen preparation.

2 cc 4% whey preparation were used, the concentration of the hypoxanthine solution was M/136, and its total volume in each tube was 0·6 cc, and the time of incubation with cyanide was 8 min at 38°; the procedure was otherwise the same.

In figs. 15 and 16 the upper line represents the control without cyanide, and the lower line is the line of zero protection. In fig. 15 the dotted curve shows approximately the results which would be obtained by substituting an equivalent amount of uric acid for the hypoxanthine in the tube (from another experiment). It appears that the hypoxanthine curve starts to follow the uric acid curve, but with higher concentrations another effect makes itself felt and causes the divergence.

Finally, it may be mentioned that inhibition is not produced either by anaerobic incubation of oxidase and hypoxanthine together, before adding the cyanide and methylene blue, or by incubation of cyanide and hypoxanthine together, before adding the oxidase and methylene blue.

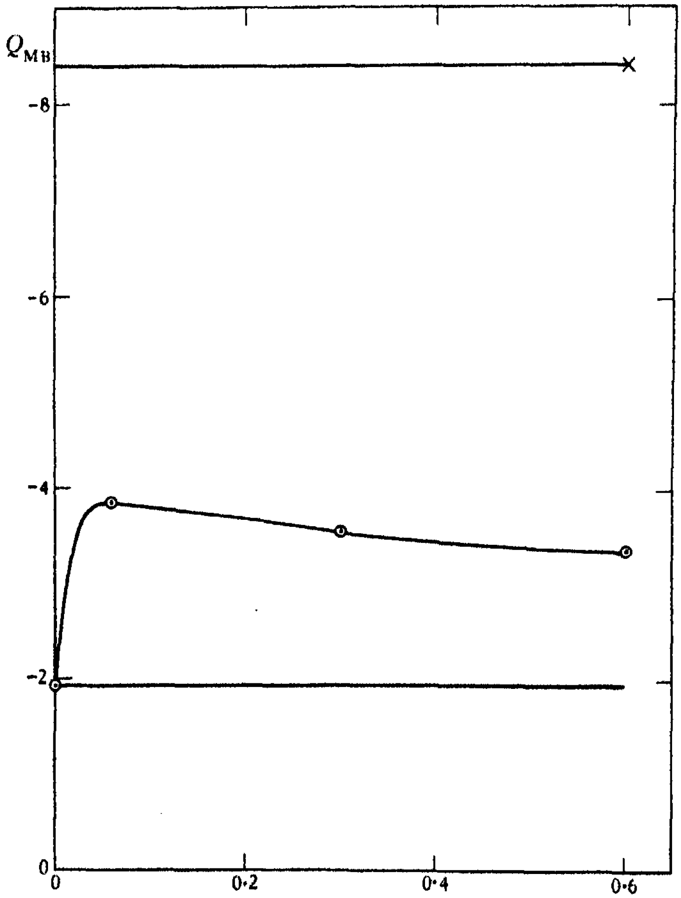


Fig. 16—Effect of hypoxanthine concentration with whey preparation.

## VI—Other Respiratory Inhibitors

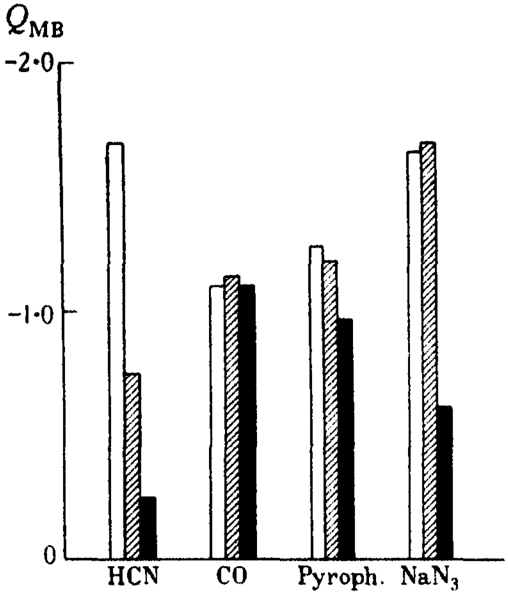
In addition to cyanide, the action of the other respiratory inhibitors (CO, $H_2S$, azide, pyrophosphate) on the xanthine oxidase was also tested. None of them, however, showed any action on the oxidase, even on prolonged incubation with it.

As it was found that the presence of hypoxanthine during the incubation with cyanide could increase the inhibition, it was thought that it might also make the enzyme more sensitive to the other inhibitors. In each case the oxidase was accordingly incubated with the inhibitor both in the presence and absence of hypoxanthine. With CO, $H_2S$, or pyrophosphate, no inhibition was obtained in either case; with azide, however, the interesting result was obtained that, while by incubating the enzyme with azide alone no inhibition whatever was produced, incubation with azide plus hypoxanthine produced a marked inhibition. Typical results obtained with methylene blue are shown in fig. 17, where the resulting activities are represented diagrammatically. The variations in the activities of the controls in the different experiments are due to the fact that a number of different enzyme solutions was used.

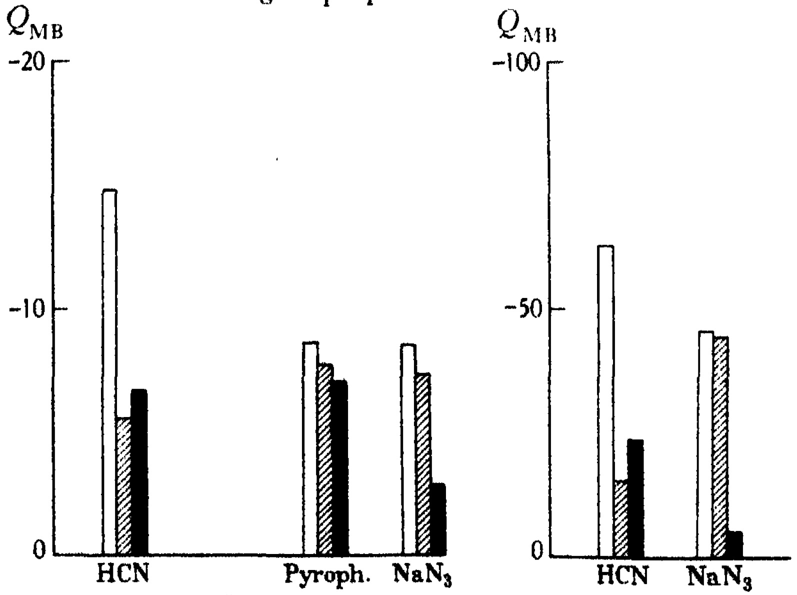
The caseinogen preparation was used in 8% solution, the whey preparation in 4%, and the Dixon and Lemberg preparation in 1%. The quantities and procedure were the same as previously described for caynide, except that with all other inhibitors the incubation was increased to ½ hour at 38°, and that 0·25 cc M/10 pyrophosphate and 0·25 cc M/30 sodium azide were taken. For the experiments on CO the Thunberg tubes were alternately exhausted and filled with pure CO twice, and were finally left filled with CO at a pressure of ½ atmosphere. Slight indications of an effect similar to the azide effect were obtained with pyrophosphate, but they are so small that we cannot say definitely that a real effect exists. The pyrophosphate concentration was high and a salt effect may be responsible.

Although the azide effect was discovered with the caseinogen preparation by analogy with the cyanide effects with hypoxanthine, it will be noticed that it also occurs with the other preparations in which hypoxanthine partially protects against cyanide. It is probable, therefore, that the two effects are independent, and the azide effect may be due to some secondary chemical reaction.

As $H_2S$ rapidly reduces methylene blue, it is impracticable to test its action by the methylene blue technique, and the oxygen uptake method must be used. For this purpose 2 cc 8% caseinogen preparation were placed in each of three manometers, and 0·24 cc M/33 $Na_2S$ and 0·5 cc (1 mg) hypoxanthine were placed in side bulbs so that they could be added later. The vessels were then completely freed from oxygen by passing pure nitrogen through them. The hypoxanthine was then introduced in manometer 1 (the control without $H_2S$), the $Na_2S$ in manometer



Caseinogen prep.



Whey prep.

Dixon and Lemberg prep.

FIG. 17—Action of various inhibitors. ☐ Control without inhibitor; ▨ incubated with inhibitor alone; ■ incubated with inhibitor + hypoxanthine.

2, and the $Na_2S$ and hypoxanthine together in manometer 3. They were then shaken for 20 min at 38°, after which the hypoxanthine was added to manometer 2, 500 cc air were quickly passed through each flask, and the oxygen uptake was followed manometrically. The results are shown in fig. 18. Similar results were also obtained in another experiment in which the incubation time was prolonged to 1 hour.

It will be seen that there has been no inactivation of the oxidase, but that the $H_2S$ produces another effect, namely, a doubling of the oxygen uptake. This is due to a coupled oxidation of the $H_2S$ by the peroxide formed during the oxidation of the hypoxanthine, half the oxygen taken up being used in this latter reaction and the other half in the oxidation of the $H_2S$.

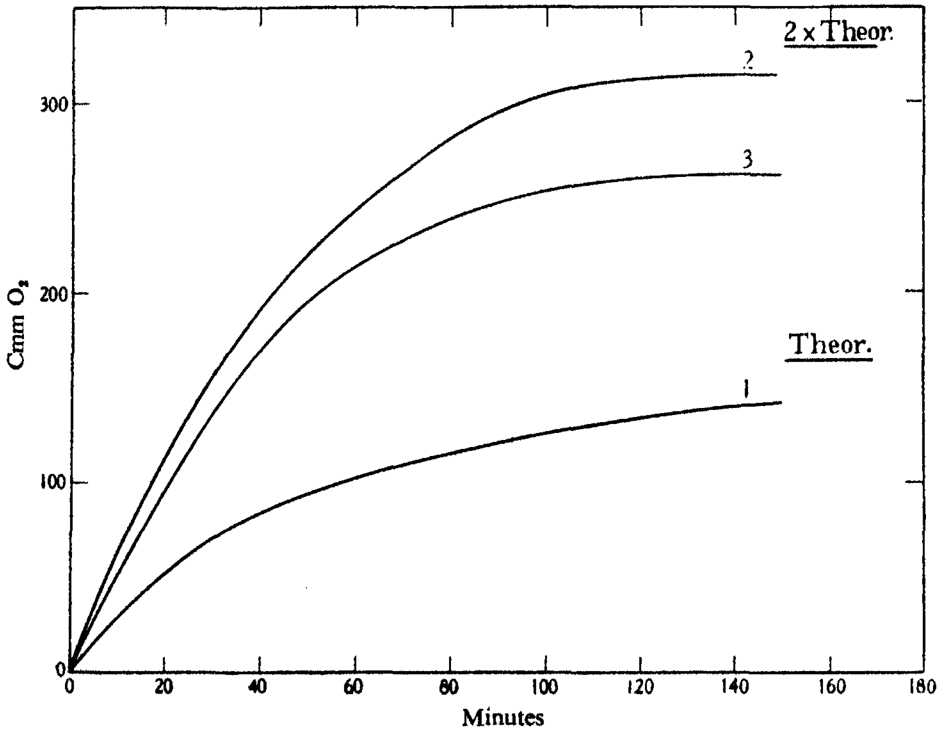


FIG. 18—Action of $H_2S$.

Curves showing that CO does not inhibit the oxygen uptake of the xanthine oxidase system have already been published by Dixon (1927).

## VII—IDENTITY OF XANTHINE OXIDASE AND SCHARDINGER ENZYME

If, in the experiment illustrated in fig. 1, acetaldehyde is used as substrate in the place of hypoxanthine the same results are obtained. In other words, the Schardinger dehydrogenase undergoes the same unusual type of inactivation by cyanide as the xanthine dehydrogenase. Moreover, the rate of inactivation is the same in both cases: when 50% of the xanthine oxidase has been destroyed so also is 50% of the Schardinger enzyme.* In view of the fact that no other anaerobic oxidases have been found to be affected by cyanide, this may be regarded as strong evidence in favour of Dixon and Thurlow's (1924, *b*) suggestion that the two enzymes are identical (*see also* Booth, 1935).

The protective effect of uric acid mentioned above gives us a means of testing this suggestion. It is clear that uric acid and cyanide both combine with the catalyst which is responsible for the activation of hypoxanthine. Now it has been mentioned above that the cyanide also reacts with the catalyst which activates aldehydes, and the uric acid effect therefore gives us a method of determining whether these two are in fact one and the same, *i.e.*, whether the two oxidases are identical. For if the two catalytic centres are identical, the aldehyde centre should also be protected from inactivation by cyanide by the presence of uric acid; while if the two enzymes are different there is no reason why the aldehyde centre, as well as the hypoxanthine centre, should be protected by uric acid.

In figs. 19 and 20 are shown the results obtained with oxygen and methylene blue respectively. The experiment shown in fig. 19 was carried out in the same way as that of fig. 10, except that 0·5 cc 10% acetaldehyde was substituted for the hypoxanthine, and the curves are numbered so as to correspond. (The quantities used were, however, different, namely, 100 mg whey preparation and 2 mg uric acid, incubated 10 min with M/500 cyanide.) Curve 3 practically coincides with curve 1, the control, showing that the aldehyde oxidase, like the xanthine oxidase, has been almost completely protected by the uric acid. Curve 4, which coincides with curve 1, was obtained by adding the cyanide and aldehyde (without uric acid) simultaneously at the beginning of the experiment. The protection in this case is, however, probably not due to the same cause as the protection by hypoxanthine previously obtained, but to the removal of the cyanide by the formation of a cyanhydrin with the aldehyde. This explanation cannot, however, apply to the protection shown in curve 3, as in this case the aldehyde was not added until after the period of incubation with cyanide.

---

* This is, however, only true if the methylene blue technique is used for testing the activities. The ability of the enzyme to reduce methylene blue in presence of hypoxanthine or of aldehyde and its ability to take up oxygen in presence of hypoxanthine all fall off at the same rate under the action of cyanide. Its ability to take up oxygen in presence of aldehyde is, however, much less affected by cyanide. For instance, in the experiment shown in fig. 1, after 2 hours' incubation with cyanide the oxidase had practically completely lost its power of reducing methylene blue in the presence of acetaldehyde ($Q_{MB} = -0·03$) and also its power to oxidize hypoxanthine either aerobically or anaerobically. Its power of oxidizing acetaldehyde aerobically was, however, only inhibited by about 50% ($Q_{O_2} = -0·8$). This remarkable effect, which has been repeated several times, cannot yet be explained and requires further investigation.

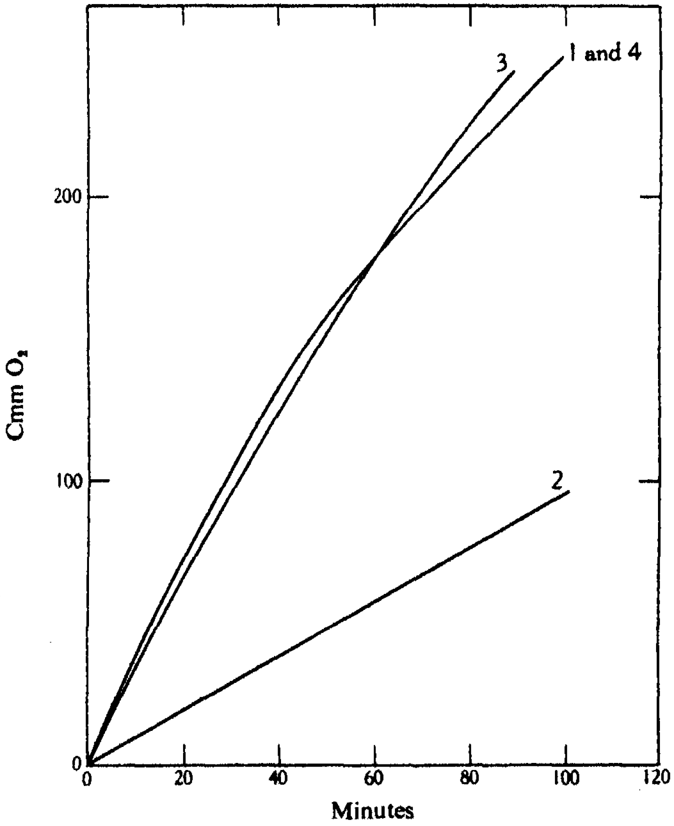


FIG. 19—Protection of Schardinger enzyme by uric acid.

The results with methylene blue are shown in fig. 20, which may be compared with fig. 12, as it was carried out at the same time and with the same enzyme solution. The quantities and procedure were exactly the same in the two cases, except for the substitution of 1 cc 0·05 M acetaldehyde for the hypoxanthine. In this case the activity without uric acid or cyanide was $Q_{MB} = -0{\cdot}68$, thus lying outside the scale of the figure. The upper line shows the activity with uric acid but without cyanide. It will be seen that here again uric acid protects the aldehyde oxidase, and, moreover, that the relative degree of protection with any given concentration of uric acid is approximately the same as in the case of the xanthine oxidase. These facts, in our opinion, form the most convincing evidence yet put forward for the identity of the xanthine oxidase and the Schardinger enzyme.

## VIII—DISCUSSION

It is by no means easy to interpret the admittedly complex group of facts given below. It is clear that we have here a new type of cyanide inhibition, differing in several respects from the type usually found with other oxidation catalysts. The usual type of inhibition, which affects the oxygen uptake only, is produced instantaneously, is readily reversible, and is only partial, a definite percentage inhibition corresponding with each cyanide concentration. In this case, on the other hand, the inhibition is produced by a relatively very slow reaction, is apparently quite irreversible, and is ultimately complete at all cyanide concentrations. Moreover, the reduction of methylene blue and the uptake of oxygen are affected equally.

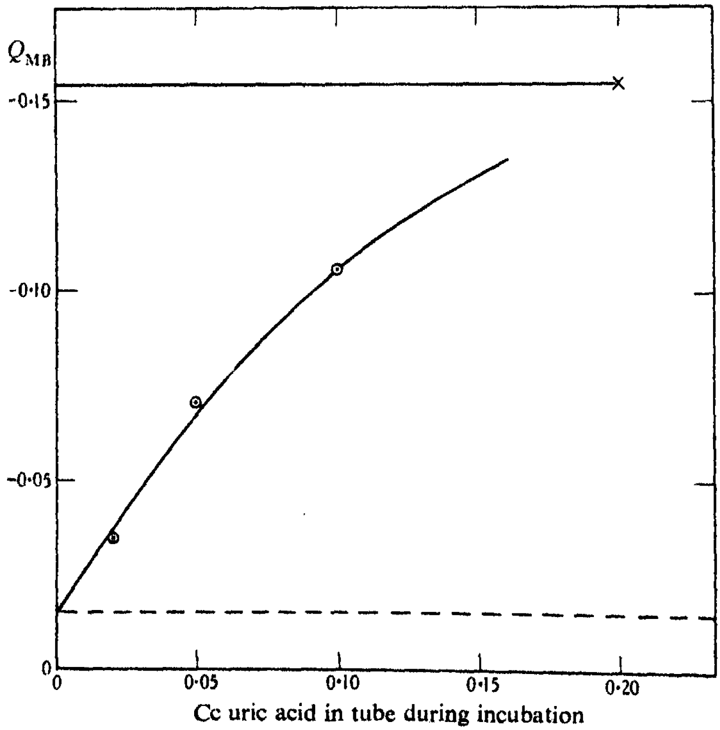


FIG. 20—Effect of uric acid concentration with Schardinger enzyme.

This last point is of importance, as it shows that it is the dehydrogenase itself, and not some oxygen-activating catalyst associated with it, which is poisoned by cyanide. It is the activation of the hypoxanthine which is interfered with by cyanide; and the effects observed therefore afford no evidence of oxygen-activation.

In our opinion, it is not possible to draw any definite conclusion as to the chemical nature of the oxidase from the facts given in this paper; from the cyanide effects alone it would clearly be unsafe to conclude either that the oxidase is or is not a metal compound.

The protective action of purines satisfactorily explains the puzzling apparent discrepancy between Dixon and Thurlow's results and later work. The results obtained depend upon the order in which the reagents are added to the oxidase. If the cyanide and hypoxanthine are added approximately simultaneously, as in Dixon and Thurlow's experiments, the enzyme is protected against the action of cyanide, while if the cyanide is added some time before the hypoxanthine the enzyme is inhibited.

The phenomenon of protection of the enzyme against cyanide by purines forms an instance of an effect fairly frequently met with in the study of enzymes, namely, the protection of an enzyme against a destructive agency by the presence of its substrate. It is undoubtedly due primarily to a blocking of the active part of the enzyme by the substrate so as to render it inaccessible to the poison. Nevertheless, a simple explanation is rendered difficult in this case by the difference in behaviour between hypoxanthine and uric acid or other purines. It appears that protection by hypoxanthine is only obtained when the hypoxanthine and the hydrogen acceptor are simultaneously present. It has been shown that in anaerobic incubation with cyanide and hypoxanthine at most only a slight degree of protection is obtained, and in fact the reverse effect may be found. No protection is shown on incubation with cyanide and the hydrogen acceptor: this is shown for methylene blue by the experiment on p. 166, and for oxygen in the experiments of figs. 1 to 4, in which the incubation was done in open vessels. Thus, although neither the hypoxanthine alone nor the hydrogen acceptor alone can confer protection, complete protection is obtained when both are present together, or in other words while the oxidation of hypoxanthine is actually proceeding. Uric acid, adenine, and guanine, however, give protection by themselves, and do not require the presence of the hydrogen acceptor. This might suggest that the protection observed with hypoxanthine is in fact due to the uric acid resulting from its oxidation, but this protection is complete even in the early stages of the reaction, when the amount of uric acid present is small, and if added to the oxidase directly would confer only relatively slight protection. An adequate explanation of these facts must await further investigation.

In view of the facts given in this paper, it is desirable that other enzymes should be investigated by similar methods. It is possible that other oxidases which have been thought to be resistant to cyanide may become inactivated when incubated with cyanide in the absence of their substrates.

We wish to express our indebtedness to Dr. E. F. Hartree, who gave us assistance in a number of the experiments.

## IX—SUMMARY

Xanthine oxidase is inactivated on incubation with cyanide. This inactivation, however, differs from the usual type in that it is produced relatively slowly, is irreversible, and affects both the anaerobic and aerobic oxidations.

Aerobically the enzyme is protected against cyanide by the presence of purines. Thus cyanide added during the oxidation of hypoxanthine produces no inhibition.

Anaerobically uric acid, adenine, guanine, etc., also protect the enzyme against cyanide. Hypoxanthine, however, only protects the enzyme appreciably in the presence of a hydrogen acceptor. In the absence of the latter it may actually increase the effect of cyanide.

No inhibition is produced by CO, $H_2S$, pyrophosphate, or $NaN_3$, even on incubation with the enzyme. On anaerobic incubation with the enzyme in presence of hypoxanthine, however, $NaN_3$ (but not CO, $H_2S$, or pyrophosphate) produces a large inhibition.

Strong evidence is given for the identity of xanthine oxidase with the Schardinger enzyme.

## REFERENCES

Booth (1935). 'Biochem. J.,' vol. **29**, p. 1732.
Dixon (1925). 'Biochem. J.,' vol. **19**, p. 507.
—— (1927). 'Biochem. J.,' vol. **21**, p. 1211.
Dixon and Kodama (1926). 'Biochem. J.,' vol. **20**, p. 1104.
Dixon and Thurlow (1924, *a*). 'Biochem. J.,' vol. **18**, p. 971.
—— (1924, *b*). 'Biochem. J.,' vol. **18**, p. 976.
—— (1925). 'Biochem. J.,' vol. **19**, p. 672.
Green and Dixon (1934). 'Biochem. J.,' vol. **28**, p. 237.
Harrison and Thurlow (1926). 'Biochem. J.,' vol. **20**, p. 217.
Keilin and Hartree (1935). 'Proc. Roy. Soc.,' B, vol. **119**, p. 114.

612 . 172 . 2

# Respiratory Variations of the Heart Rate

## I—The Reflex Mechanism of the Respiratory Arrhythmia

By G. V. Anrep, F.R.S., W. Pascual, and R. Rössler

(From the Physiological Laboratory, University of Cairo, Egypt)

[Plates 7 and 8]

The fact that under certain conditions in some animals and in man inspiration is accompanied by an acceleration of the heart rate has been demonstrated by many observers and can be considered as well established. Agreement has not yet been reached, however, about the mechanism underlying this respiratory cardiac arrhythmia. E. Hering (1871), H. E. Hering (1895), Nikiforovsky (1910), Blumenfeldt and Putzig (1914) regard the arrhythmia as caused by a reflex the stimulus for which originates within the lungs. Traube (1865), Frédéricq (1882), Foa (1913), Snyder (1915), and Heymans (1929) attribute it to a purely central influence; Bainbridge and Hilton (1918–19), and Bainbridge (1920) deny any direct influence of the respiratory act upon the heart rate and ascribe the arrhythmia to the rise in the intra-auricular blood pressure taking place during inspiration. Thus Bainbridge regards the respiratory changes of the heart rate as a reflex which accompanies respiration but is not directly caused by it. Recently H. E. Hering accepted this point of view (1933). The question whether inspiration provides the stimulus for the cardiac acceleration or whether the expiration provides the stimulus for the retardation or both is not yet settled. Neither is there an agreement about the efferent path through which the changes in the heart rate are produced, the effect being attributed by some authors to variations of the vagus tone and by others to variations of the accelerator tone. The present investigation was undertaken in order to obtain further information upon the subject and, if possible, to find the cause of the difference between the observations of the previous authors. The research was begun in 1929 in the Physiological Laboratory of Cambridge and continued up to the present in Cairo.

Our preliminary experiments convinced us that there were at least two distinct mechanisms underlying the respiratory cardiac arrhythmia, a reflex and a central mechanism. It was also soon found that the result of action of these two mechanisms may be greatly changed or even reversed under different conditions. The reflex and the central mechanism may overlap or interfere with each other so that the study of one of them in the presence of the other is confusing and unreliable. Our investigation thus became naturally divided into three sections: (*a*) the study of the reflex mechanism in absence of the central mechanism, (*b*) the study of the central mechanism in absence of the reflex mechanism, and (*c*) the study of the interaction between the reflex and the central mechanisms. Only reaction (*a*) is considered in Part I; sections (*b*) and (*c*) are discussed in Part II.

## METHOD

In view of the great dependence of the respiratory arrhythmia on the vagus tone, we had to resort to such methods as would give us a complete control over the factors known to affect it, such as the arterial blood pressure, the output of the heart, and the composition of the circulating blood. Therefore, the majority of the experiments were performed on the " heart lung head " preparation and on the " innervated heart lung " preparation. In addition, a large number of observations was made on the whole animal. Dogs were used in all experiments.

The essential difference between the two preparations is that in the first all the vascular communications between the heart and the head of the animal are left intact, the head being supplied with blood by the heart of the same animal. In the second preparation the head is supplied with blood from another source so that the thoracic and the cephalic circulations are separated and changes in the mechanical conditions of the thoracic circulation cannot directly influence the perfused head. In most experiments with the latter method the head was perfused by means of a second heart lung preparation. A diagrammatic illustration of the circulation in the two preparations is given in fig. 1.

The heart lung preparation is established by short-circuiting the upper part of the descending aorta with the vena cava inferior, the blood from the aorta being passed through an ordinary heart lung apparatus consisting of an adjustable resistance, a venous reservoir, and an arrangement for warming the blood. In the innervated heart lung preparation, in addition to the above, the peripheral ends of the left subclavian artery and of the brachiocephalic artery are connected with the arterial side of another heart lung preparation while the vena cava superior is used for the draining of the blood returning from the head into the venous reservoir of the preparation perfusing the head. The azygos vein and all the visible branches of the subclavian arteries must be ligatured.

Both preparations have been in use in this laboratory for many years; they are not difficult to perform and with comparatively little care they can be kept in an excellent condition for many hours. They present to our minds a considerable advantage over the method of intercarotid crossed circulation. The internal carotid arteries in the dog are small and at the same pressure they carry considerably less blood than the vertebral arteries. The perfusion through the four arteries ensured a good supply of blood to the brain of about 80 cc per minute at a mean pressure of 110 mm Hg in dogs weighing 7 to 8 kg. In the innervated heart lung preparation there is always a small interchange of blood between the head and the thorax. On several occasions the extent of this interchange was tested by injection into the cephalic circulation of

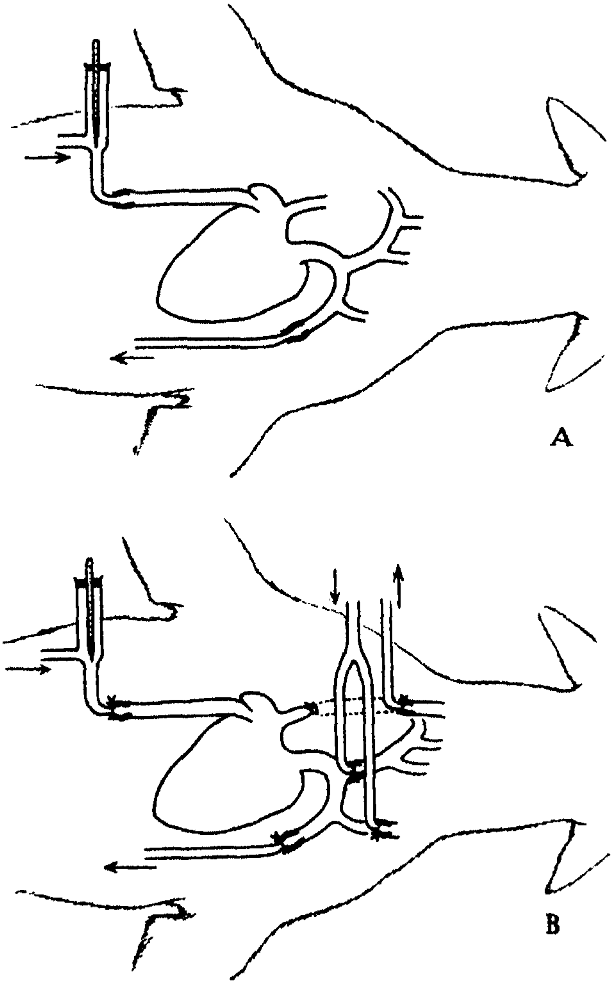


FIG. 1—Diagram of the heart lung head preparation A and of the innervated heart lung preparation B. The aorta and the inferior vena cava are short-circuited in both preparations, the blood being made to pass through a heart lung apparatus which is not shown in the diagrams. In the second preparation the head is perfused with blood from an independent source, which in most experiments was a denervated heart lung preparation.

adrenaline, atropine, or of congo red and estimating the appearance of these substances in the thoracic circulation or *vice versa*. Injection of adrenaline (after section of both vagi) into the head is not followed by an acceleration of the heart unless the dose of adrenaline is very large. Injection of adrenaline into the thoracic circulation fails to produce vaso-constriction in the head. A small dose of atropine when injected into the head, although it leads to an immediate paralysis of the chorda tympani, paralyses the cardiac vagus only after an interval of a few minutes. Congo red injected into the head appears in the thoracic circulation in about 8 to 10 minutes. Thus although a certain interchange of blood is present it is not large enough to vitiate the results of our experiments. From the point of view of purely haemodynamic conditions, the cephalic and the thoracic circulations can be regarded in the innervated heart lung preparation as almost completely separated from one another.

The results obtained by means of the two preparations were in all essential points identical, therefore they will be described together.

The heart rate was recorded electro-cardiographically, the leads being taken from the right auricle and from the apex. The lungs were ventilated with the " Ideal " respiratory pump and a rubber membrane tambour was connected with the tracheal cannula in order to record the ventilation. Since the " Ideal " pump is provided with sliding valves the tambour recorded the pressure of air throughout the whole period of inflation and not merely the rush of air in and out of the lungs as happens with ordinary pumps. The deflation of the lungs was passive, that is, it was due to their natural elasticity. The activity of the respiratory centre was registered by an indirect method, namely, by recording the movements of the larynx. The lever of a large tambour with a loosely stretched rubber membrane was stitched or glued to the larynx. The tambour was connected by air transmission to a small piston recorder, the movements of which were photographed on the same film as the electro-cardiogram and the movements of the lever registering the ventilation of the lungs. Although this and similar methods of registration of the respiratory activity were used before, the degree of their accuracy has never been determined. For our purpose it was obviously of great importance to know to what extent the laryngeal movements represent the inspiratory activity of the respiratory centre. The necessary tests were performed with the kind help of Professor E. D. Adrian, F.R.S., and Dr. D. W. Bronk, to both of whom we wish to express our sincere thanks. It was thought that no better criterion of the inspiratory activity could be used than the electrical deflexions of the central end of the cut phrenic nerve. These were recorded simultaneously with the mechanical registration of the laryngeal movements. The amplification of the phrenogram was such that a sufficiently exact determination of the beginning and end of the inspiratory volleys to the diaphragm could be made, fig. 2, Plate 7. The result of 43 determinations is given in Table I.

It follows from these measurements that the electrical deflexions of the phrenic nerve begin slightly before the laryngeal movement and end slightly after the onset of the laryngeal relaxation. The average discrepancy is in both cases about 0·05 second, so that the activity of the respiratory centre as measured in our records of the laryngeal movements appear to be 0·1 second shorter than when measured by the duration of the electrical deflexions of the phrenic nerve. Whether this discrepancy is a real one or due to instrumental errors it is too small to introduce an appreciable error in our research since the shortest cardiac cycle observed in our experiments was 0·32 second. The objection may be raised that the phrenic deflexions themselves do not accurately represent the activity of the respiratory centre as a whole. This we have no way of testing, but seeing that laryngeal movements and the phrenic deflexions, representing the activity of widely different groups of respiratory muscles, correspond so closely, the objection cannot be a serious one. It is necessary to add that between the volleys descending along the phrenic nerve during inspiration, that is, during the period of quiescence of the respiratory centre, the nerve is by no means quiet. Small electrical deflexions can be observed in the nerve during the whole period of expiration. These small deflexions cannot, however, be confused with the true inspiratory discharges.

TABLE I—COMPARISON OF THE ELECTRICAL AND MECHANICAL REGISTRATION OF RESPIRATION

| | Extreme differences in seconds | Average differences in seconds |
|---|---|---|
| Electrical deflexions begin before the laryngeal movements by .......... | 0·03–0·08 | 0·049 |
| Electrical deflexions end after the beginning of the laryngeal relaxation by .............................. | 0·02–0·08 | 0·045 |
| Electrical deflexions end before the termination of the laryngeal relaxation by ............................ | 0·11–0·17 | 0·142 |

The activity of the respiratory centre was controlled by varying the gas mixture used for the ventilation of the lungs of the heart lung head preparation or of the heart lung preparation perfusing the head of the experimental animal. When room air was used the dog soon became acapnic, the carbon dioxide of the arterial blood dropped to 20–30 volumes per cent., and the respiration stopped. This was shown by the absence of contraction of all visible respiratory muscles and by the absence of the respiratory electrical deflexions of the phrenic nerve. It was necessary to administer air containing 2·0 to 3·5% carbon dioxide in order to maintain the activity of the respiratory centre at approximately the same intensity which it had before the preparation was set up.

## THE REFLEX NATURE OF THE ARRHYTHMIA

The first description of the respiratory changes of the heart rate was given by Ludwig (1847). On analysing the changes Einbrodt (1860) found that inflation of the lungs through the trachea usually caused a *slowing* of the heart. This slowing was more conspicuous at the higher ranges of the inflation pressure which in most of Einbrodt's experiments varied between 25 and 150 mm of mercury. E. Hering (1871) objected to these experiments on the ground that the slowing of the heart might have been due to various reflexes indirectly brought about by subjecting the lungs to such abnormally high pressures. Hering found that when the lungs are inflated with moderate pressures, not exceeding about 15 mm of mercury, the effect is one of *acceleration* of the heart. This acceleration could be observed in animals with opened or closed chest, and it was unaffected whether the volume of the lungs was increased by intratracheal insufflation or by lowering the intra-thoracic pressure. Section of the cervical vagi abolished the effect of the inflation of the lungs. The researches of Hering did not extend beyond the mere statement of the fact, and the work of the subsequent observers did not advance the analysis of his observations much further. In fact, the reflex theory of the cardiac arrhythmia was soon discarded and replaced by the central theory which is at present that generally accepted.

The observation of E. Hering that section of the cervical vagi abolishes the cardiac effect of pulmonary inflation does not necessarily prove that the afferent path of the reflex runs along these nerves. The disappearance of the reflex may be due to severance of its efferent path or to the fact that after double vagotomy the heart beats so fast that the pulmonary inflation is incapable of accelerating it still further. At present there is even no conclusive evidence that the cardiac acceleration is due to impulses originating in the lungs and not in some other organ indirectly affected by the inflation. Furthermore, it is not certain whether it is the inflation or the deflation which is the operative factor in the reflex changes of the heart rate. While the followers of the central theory of the respiratory arrhythmia from Frédéricq to Heymans deny any effect of pulmonary ventilation, Nikiforovsky (1910), working in Pavlov's laboratory, believes that inflation of the lungs causes a reflex cardiac acceleration due to an excitation of the sympathetic nerve and deflation of the lungs a reflex cardiac slowing through an excitation of the vagus nerve. Lately Heymans seems to have accepted the fact that the lungs also play a role in the arrhythmia (Heymans, Bouckaert, and Samaan, 1934). H. E. Hering (1930) states that inflation itself evokes two reflexes, one accelerating and the other slowing the heart rate. Either of these reflexes may predominate, so that it is difficult to predict the result which pulmonary inflation may have on the heart. According to H. E. Hering, the slowing is more easily obtained with strong inflation of the lungs. Nikiforovsky considers that the afferent path of the reflex runs in the sympathetic fibres as well as in the vagi. Thus at the onset of our investigation of the reflex mechanism of the arrhythmia, we were confronted, on the one hand, by a complete denial of its existence by one group of observers and, on the other hand, by a mass of conflicting statements made by the workers who accept its existence.

The fact that the heart accelerates during a moderate inflation of the lungs could be observed in most of our experiments. In confirmation of E. Hering, we found it immaterial in what way the inflation was produced and whether the chest of the animal was open or closed. The following experiments may serve as an example:

Dog 5 kg; morphine 5 mg and chloralose 0·06 gm per kg. The whole animal is enclosed in an air-tight box the pressure in which can be changed. The trachea of the animal is connected with a respiratory pump outside the box. During periods of deflation the heart rate varied between 80 and 85 beats per minute; during periods of inflation through the trachea by 120 cc of air the heart accelerated to 130 beats per minute. A similar inflation of the lungs produced by diminishing the pressure in the box accelerated the heart to 128 beats per minute. The chest of the animal was then opened. This was done during the deflated state of the lungs. The amount of air expelled by the collapse of the lungs was 48 cc. The dog was then again placed in the box and the experiment repeated. Positive and negative inflation of the lungs by 170 cc (120 + 48 cc) led to the same degree of acceleration as when the chest was closed.

In order to avoid any effect on the heart rate, which the activity of the respiratory centre may have independently of the state of the lungs, all our subsequent experiments upon the reflex mechanism of the respiratory arrhythmia were made in absence of respiration. The respiration was stopped, as has been described before, by supplying the head of the experimental animal with blood containing a diminished amount of carbon dioxide. A moderate inflation of the lungs of an animal, the respiratory centre of which is in abeyance, is also accompanied by an acceleration of the heart. The degree of this acceleration varies a great deal under different conditions. In the first place it depends on the extent of the vagus tone.

### THE DEPENDENCE OF THE ARRHYTHMIA ON THE VAGUS TONE

The cardiac acceleration in response to pulmonary inflation is more pronounced at a medium intensity of the vagus tone. It has been noticed by several observers that the arrhythmia becomes more evident and sometimes makes its appearance only after administration of morphine. We can confirm this statement. It is, however, only small doses of morphine which accentuate the effect. Large doses lead to a nearly uniformly slow heart rate. The vagus tone becomes so strong that the heart rate hardly changes during the inflation of the lungs. In our experiments the tone of the cardio-inhibitory fibres could be controlled by changing the blood pressure perfusing the head of the animal or the blood pressure in the aorta. It was therefore rarely necessary to use morphine in order to accentuate the effect of pulmonary inflation; a simple rise of the arterial pressure was usually found to be sufficient.

The extent to which the changes of the vagus tone affect the cardiac arrhythmia is diagrammatically shown in fig. 3, in which the short cardiac cycles occurring during inflation of the lungs and the long cycles occurring during deflation are plotted against the arterial pressure. The extent of the effect of an increase in the vagus tone varies from one experiment to another, but the general picture is the same. The rise of the arterial blood pressure first affects the length of the " deflation cardiac cycles " and has a considerably smaller effect on the " inflation cycles " which become lengthened only with a further increase of the vagus tone. Ultimately the difference between the length of the cardiac cycles during inflation of the lungs almost disappears and the heart beats at a nearly uniformly slow rate. With low arterial blood pressure, and therefore absence of the vagus tone, the heart beats at an almost uniformly fast rate. These observations agree with the results of the experiments by H. E. Hering (1930), who found that the respiratory arrhythmia disappears after section of the depressor nerves and occlusion of both common carotid arteries so as to exclude the action of the carotid sinus. Under these conditions the vagus tone is either abolished or very considerably diminished. If, however, the vagus tone is again increased, for instance, by gently asphyxiating the animal, the respiratory arrhythmia also reappears.

The great dependence of the respiratory arrhythmia on the vagus tone shows how important it is in these experiments to control the blood pressure. In the whole animal a positive inflation of the lungs is accompanied by a fall of the arterial and by a rise of the venous blood pressure, both changes leading to an acceleration of the heart. In the heart lung preparation this effect is negligible, especially with moderate inflation of the lungs.

## THE PECULIARITIES OF THE CARDIAC RESPONSE TO INFLATION OF THE LUNGS

The cardiac acceleration in response to pulmonary inflation is not observed in heavily anaesthetized or heavily morphinized animals. It frequently disappears for a long time after administration of painful stimuli or after artificial stimulation of sensory nerves. Damages to the lungs such as those caused by a prolonged hyperaemia or by oedema rapidly diminish and finally abolish the effect. Over-distension of the lungs, even if of short duration, also leads to a temporary diminution of the effect, and if the distension is severe and is repeated the respiratory arrhythmia disappears completely.

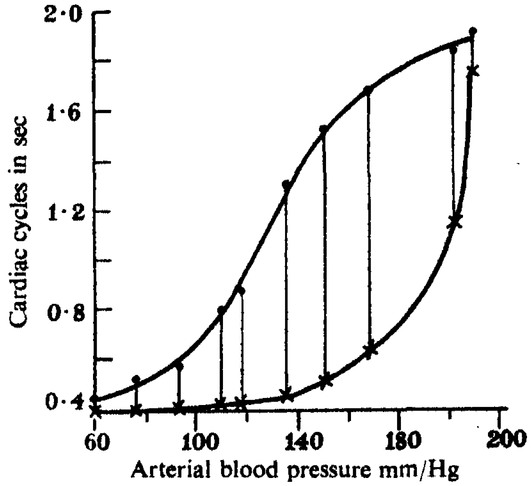


FIG. 3—The dependence of the reflex respiratory arrhythmia on the vagus tone. Abscissa—mean arterial blood pressure in mm Hg; ordinate—duration of the cardiac cycles in seconds. The shortest inspiratory cardiac cycles are indicated by crosses, the longest expiratory cardiac cycles by dots. The duration of the short and long cycles was determined by taking the mean of 10 measurements during successive inflations and deflations of the lungs. The vertical lines connecting the inspiratory and expiratory cardiac cycles for each given blood pressure show the extent of the cardiac arrhythmia.

In a fresh preparation the fast heart rate continues during the inflated state of the lungs for many minutes and conversely the slow heart rate is maintained during long periods of deflation. Such protracted changes in the heart rate in response to alterations in the state of the lungs cannot be easily observed in experiments on the whole animal in which prolonged deflation as well as inflations rapidly lead to asphyxial changes of the heart rate and of the blood pressure. The activity of the respiratory centre also begins to interfere with the reflex. In some experiments on the innervated heart lung preparation the reflex acceleration of the heart remained unchanged during a moderate inflation of the lungs which lasted up to 10 minutes. It is therefore obvious that it is not the inrush and the outrush respectively of air, and not the movements of the lungs which are the cause of the alteration of the heart rate but the inflated and the deflated state of the lungs.

In view of the fact that strong inflations of the lungs with high pressures have been reported in the literature to cause a considerable slowing and even arrest of the heart we limited our observations by employing such inflations which would not exceed the physiological range. When, after opening the thorax, the lungs are inflated with a pressure of 20 cm of water they are expanded still within physiological limits comfortably filling the chest. All our observations were made with inflation pressures below 20 cm of water. Usually the piston of the respiratory pump was set at a stroke which with a single revolution of the pump raised the pulmonary pressure to 8–9 cm and with a double revolution to 16–18 cm of water. With the lower pressure the lungs were expanded approximately to the volume which they occupy during a normal inspiration with a closed chest; with the higher pressure their volume was about equal to that reached during a strong inspiration. Obviously these figures would vary from one experiment to another depending on the bronchial tone, on the amount of blood in the lungs, and on the changes in the elasticity of the pulmonary tissue.

The maximal acceleration during inflation is reached within two or three heart beats, the development of the slowing during the subsequent deflation is, however, somewhat delayed, fig. 4, Plate 7. In consequence of this, the mean heart rate is to a considerable extent dependent on the frequency of the pulmonary ventilation. On increasing the number of pulmonary inflations per minute, the heart rate may be accelerated to an extent which indicates a complete loss of the vagus tone. This acceleration is not caused by the over-ventilation of the animal since it can easily be observed when the lungs are ventilated with a gas mixture containing an adequate amount of carbon dioxide. When the inflation of the lungs is rapid the heart rate may reach its maximum within a single cycle. Frequently, during such inflations, the first few heart beats are somewhat faster than the following ones. This effect was not, however, always observed. The extent of acceleration depends on the degree of the inflation of the lungs, but this dependence is obvious only within the smaller ranges of inflations. For example, inflations with a pressure of 3–4 cm of water invariably caused a somewhat less conspicuous acceleration of the heart than inflations with pressures of 6–8 cm. More vigorous inflations with pressures of 10, 15, and 18 cm usually cause no greater acceleration of the heart than that accompanying the moderate inflations. Inflations with still higher pressures, usually beyond 40 cm of water, produce an abrupt and conspicuous slowing of the heart rate. The results are so variable that it is impossible to suggest some definite volume of the lungs at which the heart rate is maximal. As far as our experiments go it seems that inflations which are slightly above the volume of a normal inspiration are accompanied by a cardiac acceleration which is not increased by further inflations.

The cardio-accelerator response to inflation of the lungs, although it is so very definite, presents, nevertheless, a complicated picture which could be made clear only after a great number of experiments. Even now many peculiarities of the reaction allow of alternative explanations, so that in describing the results of our work we feel it best to restrict ourselves to an exposition of the facts and to make only an attempt to piece these facts together.

### THE "SECONDARY SLOWING"

When the lungs are inflated with a low pressure of 8–9 cm of water the accelerator response continues in a fresh preparation for a long time. If the inflation pressure is slightly raised to 16–20 cm the heart rate after having reached a maximum gradually begins to fall off. The heart may ultimately slow down below the deflation rate. Inflations of this magnitude are still within the physiological limits. Towards the end of an experiment, especially if the lungs have been somewhat damaged by preceding inflations, even a small inflation by 10 cm pressure will fail to cause a prolonged acceleration of the heart, fig. 5. The heart beats faster during the first stage of the inflation but soon slows down. A further inflation temporarily accelerates the heart again but only for a short time. In some experiments this, which we may call "secondary slowing" of the heart, is equal to the slowing observed during the periods of deflation of the lungs. Frequently, however, it is even slower than the deflation rate, fig. 6.

Extremely interesting are the changes of the heart rate which take place when a sudden complete deflation of the lungs is made during the secondary slowing. A condition is present in which the heart rate during an inflated state of the lungs is as slow or even slower than that observed

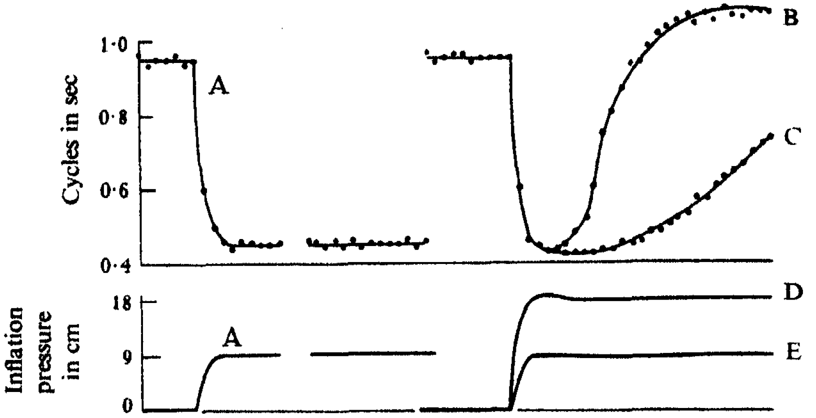


FIG. 5—Development of the " secondary slowing " during inflation of the lungs. The upper lines show the length of the cardiac cycles in seconds, the lower lines show the inflation pressure in centimetres of water.

A, effect of a protracted inflation of the lungs in a fresh preparation with a pressure of 9 cm. The interruption in the tracing is equal to 70 seconds. The fast heart rate is maintained during the whole period of inflation. B, a rapid development of secondary slowing during a period of inflation of the lungs with a pressure of 18 cm of water. The slowing of the heart exceeds that which is observed during the deflation of the lungs. C, the secondary slowing develops much less rapidly when the lungs are inflated with a lower pressure. The ultimate heart rate was the same as for the higher pressure.

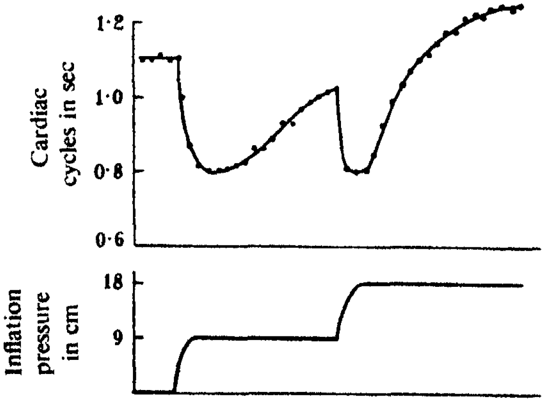


FIG. 6—The gradual development of the secondary slowing during a moderate inflation of the lungs is for a short time reversed by a further inflation. It then continues at a considerably faster rate. The maximal slowing is somewhat more conspicuous than that observed during the periods of pulmonary deflation.

during their deflation, and it is impossible to foresee what the effect would be. Fig. 7 gives the results of a few experiments which have been taken at random from a great many similar observations. It can be seen that when the heart rate during the secondary slowing is equal to that of the deflation rate, a sudden collapse of the lungs remains without an effect. The slow rate continues without any change at all. When, however, the heart rate during the secondary slowing falls below the deflation rate a collapse of the lungs is accompanied by an acceleration of the heart, but not beyond the rate at which the heart was beating during the periods of deflation. In other words, the heart always assumes the rate which is characteristic of deflation. The difference is only in the initial rate at which it has been beating before the period of pulmonary deflation. The heart slows down if the deflation occurs during the normal cardio-accelerating influence of inflation; it does not change if its rate during the secondary slowing is already equal to that observed in deflation, and it accelerates if the secondary slowing is more conspicuous than the slow rate occurring during the deflation.

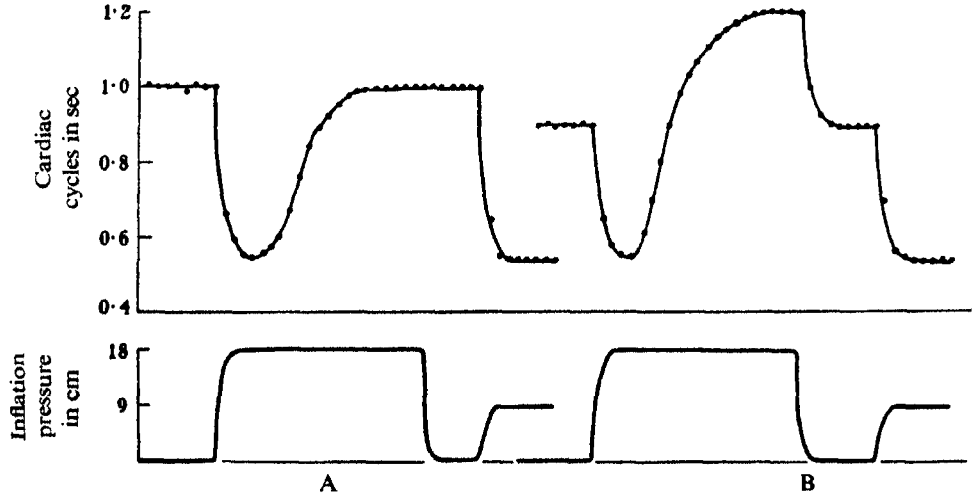


FIG. 7—*Deflation of the lungs during the secondary slowing and the recovery of the pulmonary reflex from the effect of the secondary slowing.*

A, The secondary slowing during an inflation of the lungs with a pressure of 18 cm is equal to the deflation heart rate. A deflation made during the secondary slowing causes no change in the heart rate, but a second inflation of the lungs 5 seconds later is accompanied by a normal cardiac acceleration. B, The secondary slowing is more pronounced than the slowing observed during the deflation of the lungs. A deflation made during the secondary slowing rapidly accelerates the heart to the deflation rate. A second inflation with a pressure of 9 cm is accompanied by a normal cardio-accelerator reflex.

These changes in the heart rate occur within a few beats. In some experiments the difference of the heart rate during the deflation and during the secondary slowing may be considerable, so that one can observe a sort of inversion of the normal response to deflation of the lungs. A deflation made during such a conspicuous secondary slowing leads now to a considerable acceleration instead of, as is usual under normal conditions, to a slowing of the heart rate.

Another peculiarity worthy of note is that if the inflated state of the lungs is suddenly released during the secondary slowing and then, after an interval of a few seconds, the inflation is again repeated the heart responds by a perfectly normal acceleration which, if the inflation is sustained, gradually changes again into a secondary slowing. Apparently the accelerator response to inflation which disappears during the secondary slowing recovers extremely rapidly when the lungs are deflated. The recovery may be complete in 2–3 seconds, fig. 8, Plate 8.

In many experiments the secondary slowing developed with an extreme ease. In such instances the cardio-accelerator effect of inflation is only of short duration. It occurs almost synchronously with the changes in volume of the lungs and then rapidly disappears. It is likely that this may be the reason why some observers suggested that the reflex acceleration of the heart is caused by the movements of the lungs and not by their inflated state.

## THE PULMONARY ORIGIN OF THE REFLEX RESPIRATORY ARRYTHMIA AND ITS MECHANISM

It can be seen from the above description how greatly varied may be the effect of pulmonary inflation on the heart rate. The first question which arises in the analysis of these complicated relations is whether the so-called secondary slowing is caused by some accessory factors which have nothing to do with the lungs or whether the same volume of the lungs may simultaneously bring about two antagonistic influences, one causing an acceleration and the other a slowing of the heart, the second influence gradually predominating over the first. However, before approaching this obvious question, it is necessary to consider what evidence exists to show that the afferent stimuli evoking the normal changes in the heart rate arise in the lungs and not in some other organs indirectly affected by the inflation and deflation of the lungs. Further, should these stimuli arise in the lungs, it becomes necessary to determine whether one deals in the reflex respiratory arrhythmia with an acceleration of the heart during inflation or with an inhibition of the heart during deflation. In other words, one has to determine whether the pulmonary sensory nerve endings are stimulated by the expansion or by the collapse of the lungs. The evidence relating to these questions which is found in the literature is surprisingly scanty, and the disappearance of the reflex respiratory arrhythmia after the section of the cervical vagi is repeatedly quoted as a proof of its pulmonary origin.

The crucial evidence necessary for a decision upon all these questions can be secured in experiments with section and with stimulation of the pulmonary branches of the vagus nerve.

### *The Effect of Stimulation of the Vagus Nerve in the Thorax*

The effect of stimulation of the central ends of the pulmonary nerves has been studied by Brodie and Russel (1900). These observers were interested in the reflexes which could be obtained in response to administration of various irritant vapours to the lungs, such as tobacco smoke, ammonia, and iodine vapour. They found that these substances produce a pronounced slowing of the heart rate and they looked, therefore, for a cardio-inhibitory effect of stimulation of the pulmonary nerves. In their hands stimulation of the vagus trunk below the lungs produced no change in the heart rate while stimulation above the lungs led to an arrest or to a conspicuous slowing of the heart. In describing this effect they say that on inspecting the records it is difficult to decide which was obtained by stimulation of the peripheral end of the vagus in the neck and which by the central end of the vagus above the lungs. Although it was natural to assume that this slowing is of a reflex nature, it is nevertheless surprising that these authors did not think it necessary to verify this point.

In our experiments stimulation of the central end of the thoracic vagus produced a variety of effects depending on the strength of the stimulus and on the place of stimulation. Stimulation below the lungs was ineffective so long as a reasonable strength of the stimulus was used. Any effect observed could always be traced to a spread of current. Stimulation of the vagus trunk just above the lungs and well below the cardiac fibres, when made with a very weak current, produced an immediate and conspicuous acceleration of the heart. This acceleration was usually equal to a complete disappearance of the vagus tone, which could be shown by cutting the cervical vagi during the stimulation of the central end of the vagus above the lungs. The acceleration is of a reflex nature. It is not affected by the extirpation of the stellate ganglia or of the whole sympathetic chain. It is strictly unilateral since it completely disappears after cutting the cervical vagus on the same side. The degree of the accelerations obviously depends on the intensity of the vagus tone and therefore on the height of the arterial blood pressure. For example, in one experiment while the arterial pressure was 50 mm Hg and the heart rate 180 beats per minute, stimulation of the central end of the vagus nerve just above the lungs failed to have any effect on the heart. When the blood pressure was raised to 120 mm and the heart slowed down to 60 beats per minute, the same stimulation of the thoracic vagus accelerated the heart to 180 beats and with a further rise of the blood pressure to 180 mm a heart beating at a rate of 35 accelerated to 170 beats per minute. Section of the vagi in the neck led in this experiment to a heart rate of 182 per minute. The abruptness with which this reflex acceleration of the heart makes its appearance can be seen from fig. 9. Similar accelerations of the heart could be observed during stimulation of the central ends of the fine nerves on the hilum of the lungs and also of the nerve branches running along the bronchi inside the lungs. In view of the recent publications by Barry (1935), who described cardio-accelerator efferent fibres emerging from the vagus trunk approximately at the level of the pulmonary root, it is necessary to emphasize once more that the

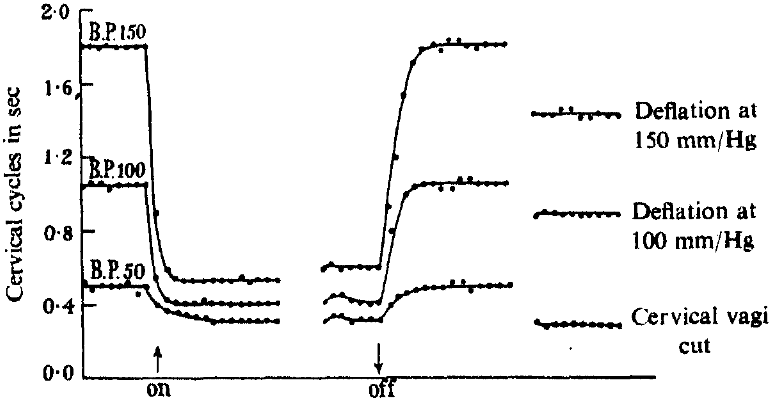


FIG. 9—The effect of stimulation of the central end of the thoracic vagus at different arterial blood pressures. The nerve was stimulated just above the right lung with a very weak current. Stimulation of the same vagus below the lungs had no effect on the heart rate. The break in the tracing is equal to 25 seconds. On the right-hand side of the tracing are given the heart rates observed during the deflation of the lungs before the pulmonary vagi were cut and also the heart rate after section of the cervical vagi. The deflation cardiac cycles are in every case shorter than those observed after the section of the vagi above the lungs at the same arterial blood pressure. The acceleration of the heart observed during the stimulation of the thoracic vagus is of the same order as that caused by section of the cervical vagi.

acceleration of the heart which we are describing cannot be attributed to a direct stimulation of the accelerator nerves since it completely vanishes after the section of the cervical vagus on the same side. Besides, it is easily obtained if the central stump of the whole thoracic vagus is dissected and separated from all the neighbouring structures and its end, at least 5 cm away from the first cardiac branch, is stimulated. In all experiments the effect is best obtained with very weak stimuli which are barely perceptible on the tongue.

Knoll in 1881 was the first to observe that stimulation of the central end of the cervical vagus with very weak currents causes a considerable loss of the vagus tone and hence a pronounced acceleration of the heart. Unfortunately, his work has completely escaped notice in modern literature. Most of the leading treatises on cardiac innervation do not even mention the observations of Knoll. Instead most text-books refer only to the fact that central stimulation of one vagus leads to a reflex slowing of the heart through the other vagus. I am indebted to Professor H. E. Hering for having drawn my attention to the communication of Knoll. H. E. Hering in 1924 repeated the experiments of Knoll and confirmed them. He also suggested that the sensory fibres of the vagus responsible for this reflex may possibly arise in the lungs. The observations described in the present communication provide the necessary proof in favour of this point of view.

Strong stimuli, confirming the results of Brodie and Russel (1900), lead to a slowing or even to an arrest of the heart. This effect consists of a reflex and of a direct component. The two can best be demonstrated if the stimulus is applied low down within the lungs and gradually advanced towards and up the thoracic vagus trunk. Strong stimuli applied to the pulmonary nerve fibres within the pulmonary tissue, on the hilum of the lungs, or just above the lungs produce a slowing of the heart which is unquestionably of a reflex nature since it disappears after the section of the corresponding cervical vagus. Stimulation below the lungs is ineffective. Usually the slowing develops gradually during the stimulation and outlasts the stimulus for an appreciable length of time. When the stimulus is applied higher up the thoracic vagus, a centimetre or so above the root of the lungs, it suddenlly becomes very much more effective, and the higher one advances along the vagus the more conspicuous becomes the slowing of the heart. It is this slowing which has been described by Brodie and Russel. Contrary to their suggestion, however, the effect is not due to a reflex, since it does not disappear after section of the vagus on the same side or even of both vagi in the neck. Neither is it due to a spread of current to cardio-inhibitory branches which may leave the vagus trunk rather low in the chest. The thoracic vagus can be laid bare for a stretch of several centimetres and the stimuli can be applied to its end about 5–6 cm below the first cardiac branch which has been left intact, but the effect of the stimulation is the same. When the nerve is stimulated after the section of the cervical vagi, the slowing of the heart appears at once, together with the application of the stimulus. After our investigation of this part of the problem was completed, Dr. E. von Saalfeld was kind enough to repeat most of these observations. His main object was to trace the origin of those fibres of the vagus the stimulation of which evokes a reflex slowing of the heart rate. In his research he obviously came across the direct effect of stimulation of the thoracic vagus which has been described above. His experiments confirmed our original observations in every respect (1932, 1933).

The two following possibilities were considered by us in the attempt to explain the mechanism of the direct effect. There may, for instance, exist bifurcations of the vagal fibres, one branch going to the heart the other descending in the thoracic trunk of the nerve so that the cardiac slowing may be due to an axon reflex. This explanation presents the difficulty that it is impossible to trace the course of these hypothetical branches. They do not descend as far as the lungs because stimulation of the finer branches on the hilum, or even stimulation of the trunk itself just above the hilum, fails to evoke this direct slowing of the heart. Neither do these fibres enter the few cardiac branches which leave the vagus below the point at which it is cut in the thorax. At least, even the strongest stimulation of their central ends fails to affect the heart rate after section of the cervical vagus. The stimulation of the central end of the vagus below the lungs is also ineffective.

The other possible explanation is that some of the cardio-inhibitory fibres may run for a distance down the thoracic vagus and then loop upwards to enter the cardiac branches. Such loops are well known to exist in peripheral nerves and the vagus itself presents a good example of them in the inferior laryngeal nerve. This possibility was tested by histological means. The vagus trunk, about 2 cm above the root of the right lung, was cut aseptically in 10 dogs. Large dogs were selected for the operation in order to facilitate the investigation to follow. Eight to 10 days after the operation the dogs were killed and the cardiac branches of the vagus above the place of the section were carefully dissected. Histological examination by the toluidine blue method and by the Marchi method showed the presence in these branches of a certain number of degenerated fibres. In addition to these observations, Dr. A. Tarkhan of our Histological Laboratory succeeded in demonstrating by means of the silver method a definite looping of some fibres in the vagus trunk. It could also be seen that not all the fibres which make up some of the cardiac branches enter them in a downward course from the central part of the vagus. Some fibres join the branch from below. Three microphotographs demonstrating these results are reproduced in fig. 10, Plate 8. We should like to thank Dr. Tarkhan for having made this investigation for us.

It is impossible to say on the basis of histological preparations if the fibres joining the branches which macroscopically appear as cardiac are actually cardiac fibres; but, taking the physiological evidence together with that provided by these preparations, it seems more than probable that it is so. We therefore believe it safe to suggest that a certain number of the cardiac fibres do not directly join the branches of the nerve going to the heart but first descend down in the thoracic trunk and then loop up again to run towards the heart. This conclusion is also supported by the fact that stimulation of the central end of the degenerated thoracic vagus produces no slowing of the heart rate. This would explain the fact why the inhibitory action of the stimulus becomes more conspicuous the higher it is applied along the central end of the thoracic vagus after the cervical vagus had been cut. The lower one descends the fewer of these loops are present. It is of interest that Brodie and Russel (1900) observed that it is much easier to bring the heart to a standstill by stimulation of the central end of the thoracic vagus below the origin of the cardiac fibres than by stimulation of the central end of the vagus in the neck. The observations described here provide an explanation of this phenomenon.

Disregarding these looping fibres which have nothing to do with pulmonary reflexes, it is now possible to conclude that weak stimulation of the pulmonary nerve fibres leads to a diminution or even to a disappearance of the vagus tone while strong stimuli may, on the contrary, increase the vagus tone. The reflex acceleration is best shown at a moderate or high tone of the vagus, while the reflex slowing can be best observed when the vagus tone is small. None of these reflexes has anything to do with the sympathetic accelerator cardiac nerves, fig. 11, A.

### *The Effect of Section of the Vagus in the Thorax*

Conclusive evidence in favour of the pulmonary origin of the reflex arrhythmia can be obtained, in our opinion, only by a section of the nerves which would completely denervate the lungs without simultaneously denervating any other organ. A comparison of the heart rate before and after such section would show whether both phases of the pulmonary ventilation or only one of them and which one is responsible for the release of the afferent impulses which reflexly affect the heart rate. If these impulses arise during inflation then a section of the nerves should abruptly slow down the heart to the deflation rate cutting out the periods of acceleration. If, on the other hand, these impulses arise during the period of deflation, then section of the pulmonary nerves should lead to a uniformly fast heart rate equal to the rate which was present during the preceding inflations of the lungs. Supposing that both the inflation as well as the deflation evoke centripetal impulses of an opposite significance, then the section of the nerves should lead to a heart rate intermediate between the two extremes. The direct method of tapping the centripetal volleys by means of an oscillograph was found to be inpracticable because of the enormous number of vagal fibres which become active during inflation and which are most probably predominantly of a respiratory significance bringing about the Hering-Breuer arrest of respiration.

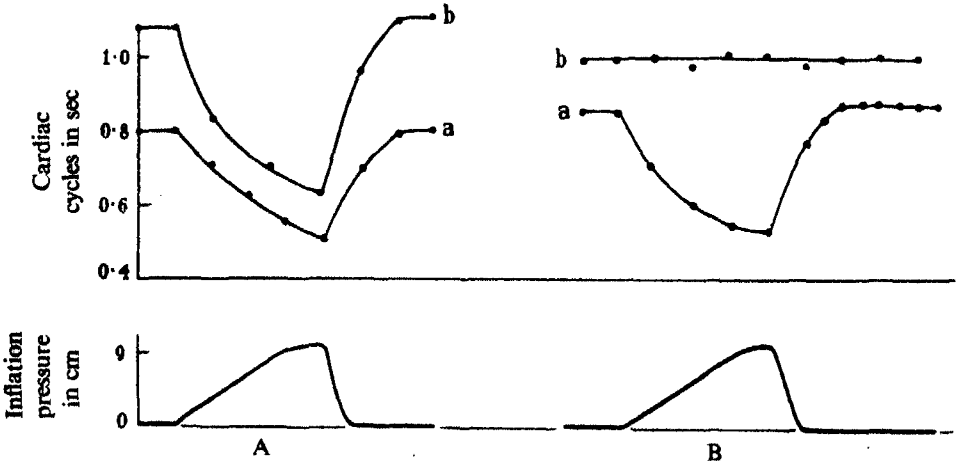


FIG. 11—A, effect of removal of the thoracic sympathetic chain including the stellate ganglia upon the reflex respiratory arrhythmia. Changes in the heart rate during a single inflation of the lungs with a pressure of 9 cm of water (*a*) before and (*b*) after sympathectomy. B, effect of section of the thoracic vagi just above the lungs. The respiratory arrhythmia disappears (*b*), and the heart rate becomes slower than it was during the periods of deflation before vagotomy (*a*).

In view of the considerable difficulties and of the uncertainty which is experienced when the section of the pulmonary nerves is attempted at the root of the lungs, we adopted the following procedure. The vagus trunks were first cut underneath the lungs so as to exclude all abdominal and the lower thoracic organs. Then a second section was made immediately above the roots of the lungs. Preliminary to this section, all the nerve filaments joining the vagus between the levels of the two sections, except those coming from the lungs, were destroyed. The second section led therefore to a denervation of the lungs without at the same time affecting the nervous connexions of the vagus with the heart.

Section of the vagi below the lungs does not modify the reflex respiratory arrhythmia. *Section immediately above abolishes it completely and the heart rate becomes entirely independent of the movements of the lungs*, fig. 11, B. It is the phase of acceleration which now disappears, the heart assuming a uniform rate which is equal to, or slower than, the rate observed during the periods of pulmonary deflation. The effect of the section of the vagi again demonstrates that the active phase of the reflex coincides with the periods of inflation. Since, however, section of the vagi just above the lungs frequently reduces the heart rate below that at which it beats during the periods of deflation (Table II), it must be assumed that, at least in some cases, a certain number of accelerator impulses are constantly emerging from the lungs and travel along the pulmonary vagal branches to the cardio-inhibitory centre. In these instances the accelerator impulses presumably only diminish during the deflation of the lungs but do not disappear altogether.

## THE PROBABLE NATURE OF THE SECONDARY SLOWING

The secondary slowing which occurs during moderate inflations of the lungs is especially marked towards the end of an experiment when the pulmonary tissue has been somewhat damaged. In fact, sometimes it becomes so conspicuous that the initial phase of acceleration of the heart may be hardly noticeable. For a few beats immediately following the inflation of the lungs the heart beats faster and then the secondary slowing sets in. The period of acceleration may be completely overlooked unless the heart rate is recorded by an accurate method.

Two possibilities could be considered in the attempt to explain the nature of the secondary slowing. It is either due to a disappearance of the pulmonary accelerator impulses or to an appearance of a new set of impulses which increase the tone of the vagus nerve. Section of the pulmonary vagi was used for the identification of the nature of the secondary slowing. In an experiment in which the secondary slowing was easily obtained the lungs were inflated with a moderate pressure (10–18 cm of water) and as soon as the acceleration of the heart gave way and its rate returned to the deflation rate or even became somewhat slower than the deflation rate, both vagi were cut just above the root of the lungs.

### TABLE II

Effect of section of the vagi just above the lungs upon the heart rate. In most experiments the vagi were cut below the lungs some time before the observations were made. The heart rate before the section of the pulmonary vagi was measured during deflation of the lungs so that the slowing of the heart produced by the section is not due to the abolition of the respiratory arrhythmia proper but to the exclusion of the "tonic" cardio-accelerator influences originating in the lungs during their collapsed state. The slowing of the heart rate is not due to the stimulating effect of the section of the nerves, because cocainization of the nerve has the same effect and because a second section made above the first causes no further change in the heart rate. Only those experiments are given here in which section of the pulmonary vagi was followed by a change in the heart rate. In the rest the deflation rate of the heart was equal to that observed after the section of the vagi above the lungs. The figures in brackets indicate the order in which the two nerves were cut.

| Heart rate during deflation of the lungs before section of the vagi | After section of right vagus | After section of left vagus | Remarks |
|---|---|---|---|
| 102 | 96 (1) | 84 (2) | |
| 120 | 84 (2) | 120 (1) | |
| 80 | 65 (2) | — | The left vagus cut before. |
| 125 | 120 (1) | 116 (2) | |
| 90 | 80 (1) | 62 (1) | |
| 80 | 53 (2) | 70 (1) | Stellate ganglia removed. |
| 90 | 75 (2) | 85 (1) | The nerves were cocainized. |
| 80 | 62 (2) | 70 (1) | Stellate ganglia removed. |
| 110 | 90 (1) | 72 (2) | Nerves are cocainized. |
| 160 | 130 (2) | 150 (1) | |
| 130 | 110 (1) | 105 (2) | |
| 160 | 60 (2) | 118 (1) | |

The last experiment presents an especial interest because before the section of the pulmonary nerves there was hardly any reflex respiratory arrhythmia present. The heart was beating at an almost uniform rate during the deflations and the inflations of the lungs. Apparently the cardio-accelerator impulses from the lungs were maximal even during the periods of deflation.

The heart rate remained unchanged. It must therefore be concluded that the secondary slowing signalizes an absence of cardio-accelerator impulses and not a presence of inhibitory ones. In other words, this means that the secondary slowing has an identical effect on the heart rate as the section of the pulmonary vagi. In fact, in the greater majority of our experiments it was found that the heart rate was in both conditions the same. The slow heart rate observed during the periods of deflation of the lungs was usually somewhat faster than that observed after the section of the pulmonary vagi. Only in a few experiments was it found to be approximately the same in all three conditions. These observations support our conclusion that even though the lungs are in a state of deflation, they may, nevertheless, exert a certain inhibitory influence upon the vagus centre.

On considering these results, it is impossible not to notice the striking similarity between the development of the secondary slowing and the phenomenon of adaptation of peripheral nerve endings. One is tempted to draw a comparison between the two and to regard the secondary slowing as an expression of adaptation of those nerve endings in the lungs which are stimulated during their inflation. At first the inflation brings about a strong volley of centripetal impulses affecting the vagus centre, but as the inflated state continues more and more of these impulses drop out and finally all of them disappear. This can hardly be due to fatigue because of the extremely rapid recovery of the effect as soon as the lungs are deflated. The recovery is complete within 2–3 seconds and possibly sooner. A small inflation is accompanied by as great an acceleration of the heart as a somewhat larger inflation, but in the first case the fast heart rate is maintained for a long time without or with a very slow development of a secondary slowing, while in the second case the acceleration is only fleeting and rapidly changes into a secondary slowing.

On investigating the action currents in fine strands of the pulmonary nerves consisting of a few nerve fibres, it could be found that some showed during a protracted inflation a fairly rapid adaptation. The majority, as claimed by Adrian (1933), have no appreciable adaptation; these are probably responsible for the Hering-Breuer respiratory effect of inflation of the lungs. The impulses which show adaptation are possibly those which are responsible for the cardio-accelerator effect. The adaptation was found to develop more rapidly when the lungs were subjected to a slightly more severe inflation than usual. However, in view of the great predominance of the slowly adaptable type of the nerve endings we had only a few observations which were successful, so that the question cannot be considered as definitely settled yet. We want to thank Dr. M. Talaat of this laboratory for the experiments upon the action currents in the pulmonary nerve fibres. The similarity between the secondary slowing and the phenomenon of adaptation is not complete. It is difficult to explain why the heart rate observed during the secondary slowing or after section of the pulmonary vagi is frequently slower than that prevailing during the deflation of the lungs. It would be expected on the basis of adaptation that the heart rate should be the same in these three conditions

## DISCUSSION AND SUMMARY

It can be seen from the description of our experiments that the influences which the lungs exert upon the heart rate are extremely complex. The work upon this problem was started about six years ago, but even at present the explanation of the various aspects of the problem rests to some extent on conjecture rather than on rigid proof. The following interpretation could be accepted provisionally; at least it covers all the facts so far discovered.

The lungs are a constant source of impulses directly inhibiting the vagus centre. This effect is a direct one and is not brought about through the intermediation of the respiratory centre because it can be observed during complete abeyance of the latter. At least it is observed in absence of all signs of activity of respiratory muscles and in absence of the respiratory deflexions of the phrenic nerve. These cardio-accelerator impulses are minimal during the deflated state of the lungs. During inflation they greatly increase and bring about a powerful inhibition of the vagus centre. The nerve endings responsible for these impulses, especially if somewhat damaged by severe or too frequent inflations, show the phenomenon of adaptation. If their damage is excessive the effect of pulmonary inflation is absent. The effect is also diminished by severe hyperaemia and by oedema of the lungs.

No evidence could be found that the stream of impulses ascending the vagus nerve from the lungs exerts any influence upon the sympathetic cardio-accelerator mechanism. Extirpation of the stellate and upper thoracic ganglia greatly accentuates the respiratory arrhythmia by abolishing the tonic influence of the accelerator nerves and therefore reducing the heart rate during the periods of deflation. The inflation rate of the heart is, however, hardly affected at all.

It was frequently observed that the inflation of the lungs may not only affect the heart rate but also the conductivity of the auriculo-ventricular bundle. In hearts, especially with a prolonged P-R interval, deflation of the lungs may be accompanied by a 2: 1 rhythm or even by a complete heart block which disappears during the periods of inflation. The respiratory arrhythmia is in such cases much less conspicuous in the auricles than it is in the ventricles.

The stimulation experiments have shown that besides the centripetal cardio-accelerator fibres the pulmonary nerves contain also some cardio-inhibitory fibres. It would therefore be expected that in addition to the accelerator phenomenon which lies at the basis of the respiratory arrhythmia the lungs must also under certain conditions act as a source of inhibitory influences. It is known, for instance, that insufflation of various irritant vapours may produce a conspicuous slowing or even arrest of the heart. It is true that at present most of these observations have to be repeated, since what has been assumed to be a pulmonary reflex may in reality be due to the chemical sensitivity of the aortic arch. No experiments have been made as yet upon the effects of such irritants before and after section of the pulmonary vagi. There is, however, one purely mechanical condition which undoubtedly leads to an excitation of the cardio-inhibitory nerve endings in the lungs. Severe pulmonary inflations which cause the lungs to bulge out of the open chest bring about a conspicuous slowing of the heart rate. With the progress of the experiments and with repetition of these forcible inflations the effect becomes considerably enhanced so that smaller inflations suffice to produce it. This inhibitory reflex can also easily be observed when the chest of the animal remains closed. The reflex completely disappears after section of the pulmonary vagi. The slowing of the heart following severe inflations has a considerable after-effect which may last for many minutes. During this after-effect normal inflations, which until now caused an acceleration of the heart, fail to have any effect at all. Apparently the accelerator mechanism has been temporarily put out of action. These cardio-inhibitory reflexes evoked by severe inflations of the lungs have been analysed in this laboratory since the completion of the present work by Dr. E. von Saalfeld (1932, 1933).

All the pulmonary reflexes can be observed after ligation of the pulmonary arteries and veins of one lung. So that any supposition that these reflexes may be caused by changes in the venous or arterial blood pressure of the animal has to be discarded.

## CONCLUSIONS

The lungs are a constant source of impulses which exert an inhibitory influence upon the vagus centre. This influence is maximal during the inflated state of the lungs and minimal, but not completely absent, during their deflation.

The nerve endings of the pulmonary afferents responsible for this influence are subject to adaptation which leads, during protracted inflations of the lungs, to the phenomenon of " secondary slowing " of the heart rate.

Proofs derived from experiments with stimulation and section of the pulmonary sensory fibres are given in support of the pulmonary origin of the cardiac effects of inflation and deflation of the lungs.

Physiological and histological evidence is provided to show that some cardio-inhibitory efferent fibres descend far down the thoracic portion of the vagus nerve where they loop up before joining the cardiac branches. These loops may be found as low down as the upper border of the azygos vein.

Section of the pulmonary vagi reduces the heart rate by abolishing the cardio-accelerating influence of the lungs. Stimulation of the pulmonary afferents inhibits the vagus centre to the point of complete disappearance of its tonic influence on the heart rate.

No evidence could be found to show that the sympathetic nerves play a role in the reflex respiratory arrhythmia.

## REFERENCES

Adrian, E. D. (1933). 'J. Physiol,' vol. **79**, p. 332.
Adrian, E. D., and Buytendijk, F. J. J. (1931). 'J. Physiol,' vol. **71**, p. 121.
Bainbridge, F. A. (1930). 'J. Physiol,' vol. **54**, p. 192.
Bainbridge, F. A., and Hilton (1918–19). 'J. Physiol.,' vol. **52**, p. 65, P.
Barry, D. T. (1935). 'J. Physiol,' vol. **84**, p. 263.
Blumenfeld, E., and Putzig, H. (1914). 'Pflüger's Arch.,' vol. **155**, p. 443.
Brodie, T. G., and Russel, A. E. (1900). 'J. Physiol.' vol. **24**, p. 92.
Einbrodt, M. (1860). 'S.B. Akad. Wiss. Wien,' vol. **40**, p. 345.
Foa, C. (1913). 'Pflüger's Arch.,' vol. **153**, p. 513.
Frédéricq, L. (1882). 'Arch. Biol. Paris,' vol. **3**, p. 55.
Hering, E. (1871). 'S.B. Akad. Wiss. Wien,' vol. **64** (2), p. 333.
Hering, H. E. (1895). 'Pflüger's Arch.,' vol. **60**, p. 429.
—— (1924). 'Pflüger's Arch.,' vol. **203**, p. 512.
—— (1930). 'Z. Kreislaufforschung,' vol. **22**, p. 593.
—— (1933). 'Arch. sci. Biol. Napoli,' vol. **28**, Proc. XIVth Int. Physiol. Congr.
Heymans, J. F., and Heymans, C. (1928). 'Arch. sci. Biol. Napoli,' vol. **12**, p. 153.
Heymans, C. (1929). 'Ergebn. Physiol.,' vol. **28**, p. 292.
Heymans, C., Bouckaert, J. J., and Samaan, A. (1934). 'Arch. int. Pharmacodyn.,' vol. **48**, p. 451.
Knoll, Ph. (1881). 'Naturwiss. Jahrbuch Lotos, Prag.,' vol. **2**.
Ludwig, C. (1847). 'Arch. Anat. Physiol.,' p. 242.
Nikiforovsky (1910). 'Proc. Military Acad. St. Petersburg,' p. 221.
Saalfeld, E. von (1932). 'Pflüger's Arch.,' vol. **231**, p. 33.
—— (1933). 'Pflüger's Arch.,' vol. **231**, p. 724.
Snyder, C. D. (1915). 'Amer. J. Physiol.,' vol. **37**, p. 104.
Traube, J. (1865). 'Z. med. Wiss.,' No. 56.

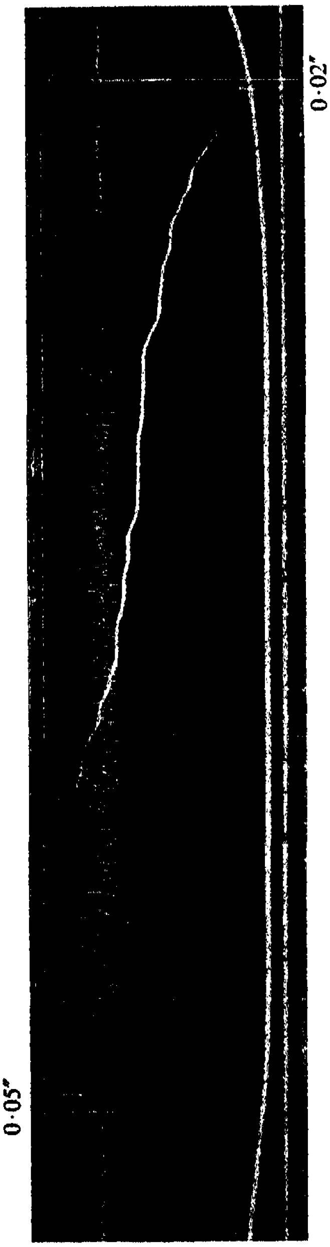


FIG. 2.

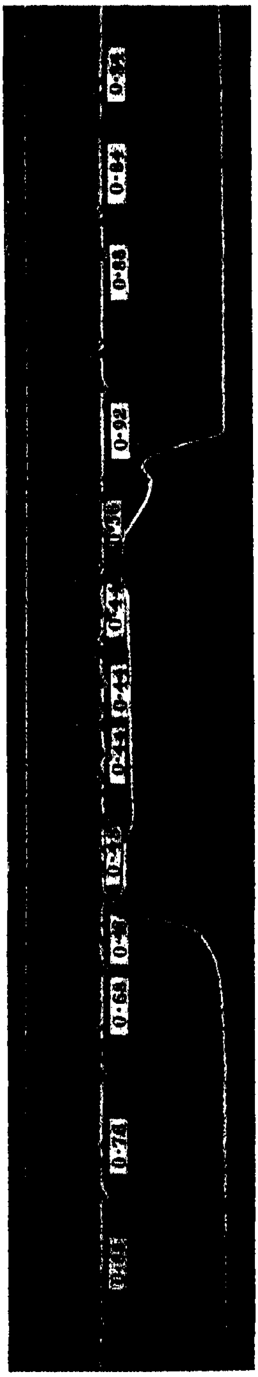


FIG. 4.

(*Facing p.* 216)

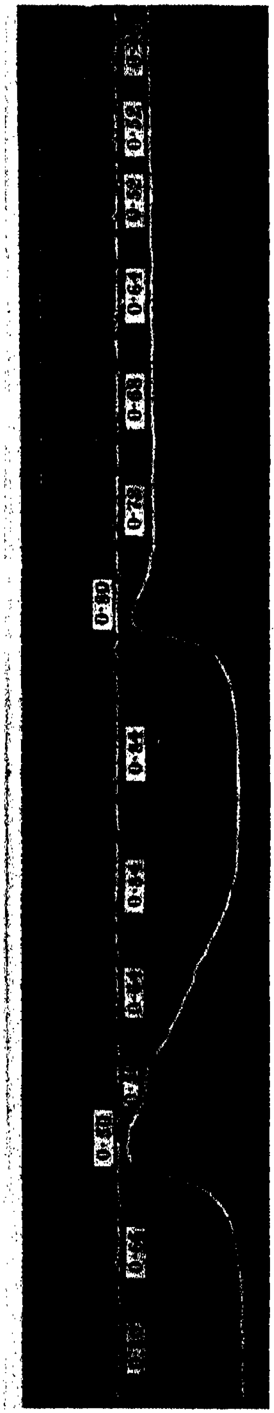


FIG. 8.

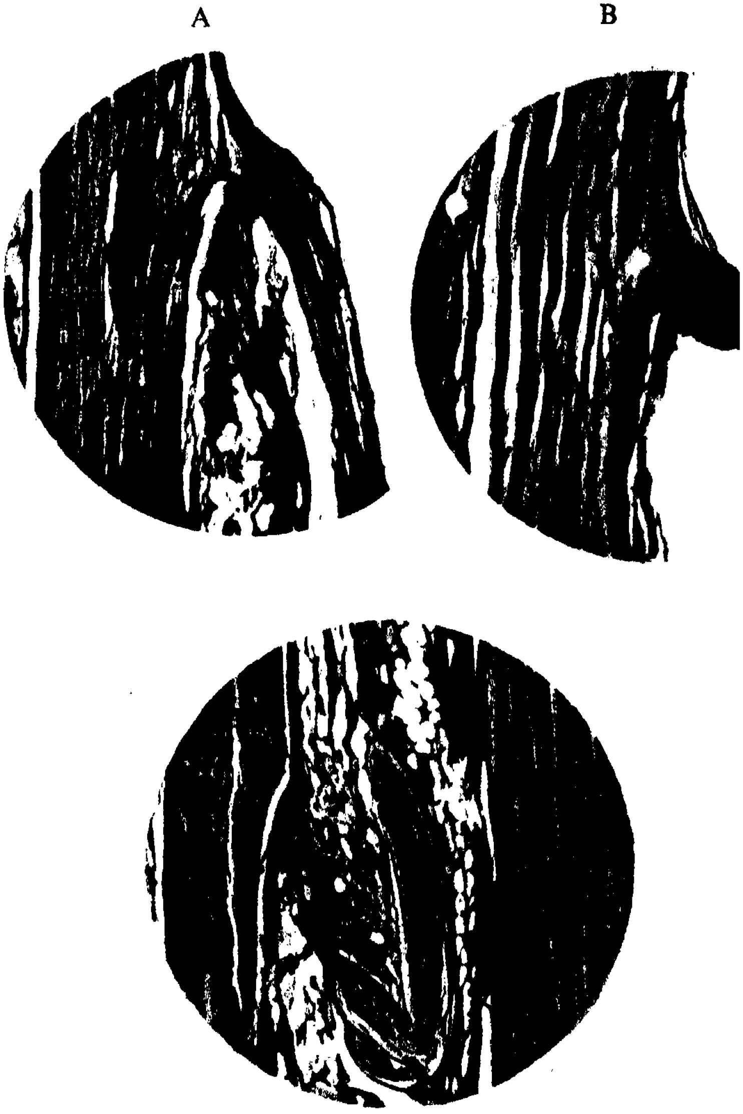


FIG. 10.

## DESCRIPTION OF PLATES

### PLATE 7.

FIG. 2—Simultaneous registration of the inspiratory movement of the larynx and of the electrical deflexions in the phrenic nerve. The two bottom lines should be disregarded. The electrical deflexions of the phrenic nerve begin about 0·02 second before the onset of the laryngeal movement and end about 0·05 second after the beginning of the laryngeal relaxation. Time in 0·04 second. The record should be read from right to left.

FIG. 4—The effect of a short inflation of the lungs on the heart rate. The inflation pressure was 9 cm of water. The heart rate is recorded by means of a string galvanometer. The length of the respective cardiac cycles is given in seconds. The maximal heart rate during the inflation of the lungs is reached in one heart beat, but the slowing down of the heart rate on deflating the lungs is gradual. In a fresh preparation the fast heart rate observed during the inflated state of the lungs continues for many minutes. The tracing should be read from right to left.

### PLATE 8.

FIG. 8—Rapid recovery from the effect of the secondary slowing. The deflation cardiac cycles were equal to 0·80–0·84 second; the inflation cycles were equal to 0·49–0·50 second. The record shows the end of a protracted inflation of the lungs with a pressure of 9 cm of water. The secondary slowing is equal to the deflation rate, hence a deflation causes no change in the heart rate. The inflation of the lungs was repeated after an interval of 1·6 seconds, the same inflation pressure being used. The cardio-accelerator reflex is found to be completely recovered. The tracing is to be read from right to left.

FIG. 10—Microphotographs of silver preparations of the thoracic vagus. A, the origin of a branch the stimulation of which produced a cardiac standstill. The nerve fibres composing this branch enter it from above as well as from below. B, an example of similar relations in another branch. C, a looping bundle of fibres in the thoracic part of the right vagus below the majority of the cardiac branches. Serial sections show that this loop connects the two parts of the nerve trunk shown on the photograph.

612.172.2

# Respiratory Variations of the Heart Rate

## II—The Central Mechanism of the Respiratory Arrhythmia and the Inter-Relations between the Central and the Reflex Mechanisms

By G. V. ANREP, F.R.S., W. PASCUAL, and R. RÖSSLER
(From the Physiological Laboratory, University of Cairo, Egypt)

(*Received October* 9, 1935)

[PLATES 9–10]

It was first noticed by Traube (1865) that the respiratory cardiac arrhythmia persists after curarization and that it becomes more obvious when the curarized animal is subjected to asphyxiation. Since, in his experiments, the muscles were paralysed and with the consequence the lungs remained in a stationary position Traube concluded that the arrhythmia could not be explained otherwise than by a direct influence of the respiratory centre upon the centres regulating the heart rate. The fact that the respiratory activity continues after administration of curare and that it becomes greatly increased during asphyxia is beyond dispute. In Traube's time this could only be conjectured, but now it can be proved by registering the electrical deflexions of the phrenic nerve or of any other inspiratory nerve. This we had occasion to verify in many experiments. The theory of Traube was for a time superseded by Hering's theory of the reflex origin of the arrhythmia (1871). However, Frédéricq (1882), in a series of masterly experiments, advanced such indisputable proofs in favour of the central mechanism that most physiologists began to doubt even the existence of the reflex mechanism. The respiratory arrhythmia continues in an animal the chest of which is widely open so that the lungs are stationary. When the respiration stops, as a result of an over ventilation, the arrhythmia also disappears. Snyder (1915) confirmed the observations of Frédéricq and expanded them by showing that the arrhythmia can be noticed even in the absence of any visible respiratory movements, but that even under these conditions it is of a central origin and depends on the respiratory centre. He suggests that the respiratory discharges may be so insignificant that they do not lead to an effective expansion of the chest; in fact, not even to a noticeable contraction of respiratory muscles, but they, nevertheless, affect the vagus centre and cause an appreciable inhibition of it. This conclusion rests on the observation that the arrhythmia occurring in absence of any sign of respiration keeps the rhythm of the respiratory movements which had been present before and which become re-established after a period of a temporary respiratory arrest. J. F. Heymans and C. Heymans (1928, 1929) investigated this problem with the help of cross circulation. They definitely express themselves in favour of the central mechanism of the arrhythmia since it is synchronous with the respiration and not with the ventilation of the lungs and since it continued, in their experiments, after the denervation of the lungs. They also found that the changes in the heart rate are not caused by the alteration of the blood pressure which accompanies the respiratory act so that the arrhythmia could not be attributed to some vascular reflexes. Although these authors find the changes in the heart rate to be synchronous with the respiration, they do not regard them as due to an irradiation from the respiratory centre since they can be observed, as has been first stated by Snyder, in the absence of all respiratory movements. Heymans (1929) suggests that there is a common rhythm which governs the activity of the respiratory and of the vagus centres and that there is no need to suppose that the respiratory rhythm directly influences the rhythm of the vagus centre. No experimental evidence has been advanced in favour of this view. Lately Heymans, Samaan, and Bouckaert (1934) seem to have accepted the fact that the lungs also play a part in the arrhythmia.

Our experiments upon the central mechanism of the respiratory arrhythmia have been carried out with the technique described in the previous paper. It is obvious that in order to study the central arrhythmia without the interfering influences arising from the pulmonary reflexes, these reflexes must be abolished. We know of three conditions in which the cardio-accelerator impulses of pulmonary origin are almost or completely stopped: during a maximal deflation of the lungs, during the " secondary slowing " occurring in protracted inflation of the lungs, and after section of the thoracic vagi just above the lungs. The last condition obviously presents greater advantages because it is inadvisable, even in the innervated heart lung preparation, to keep the lungs collapsed for a long time, and it is difficult to be sure that the secondary slowing will remain unchanged. Therefore, all our experiments were made after preliminary section of the vagi about a centimetre above the roots of the lungs.

On increasing the $CO_2$ tension in the blood perfusing the head of the experimental animal the respiratory centre begins its rhythmical activity. The heart rate increases during each period of inspiration and reaches a maximum within one to two heart beats. The termination of the period of cardiac acceleration is considerably more abrupt than it is in reflex arrhythmia, fig. 1, A, Plate 9. The termination of the acceleration coincides with the end of the electrical deflexions of the phrenic nerve. The greater the frequency of the respiration the faster, obviously, will be the average heart rate of the animal per minute. On diminishing the tension of the carbon dioxide in the circulation, the central cardiac arrhythmia diminishes *pari passu* with the diminution of the respiration. We have never found the central arrhythmia to continue in absence of respiration. It seems likely that the methods of registration of the respiration used by Snyder and by Heymans were not sufficiently sensitive. A weak respiratory effort may completely fail to be recorded by means of registration of the laryngeal contractions or of the movements of the chest. Registration of the action currents of the phrenic nerve provides a much more delicate method of measuring the respiratory cycle. On many occasions no visible contractions of the respiratory muscles could be recorded, while weak inspiratory volleys could still be detected in the phrenic nerve. In no experiment could we find a central respiratory arrhythmia in the absence of some activity of the phrenic nerve. In contrast to this we have a number of records in which the respiratory discharges were still present but were too weak to affect the heart rate. It can be observed in some experiments that the heart beats synchronously with the respiration. It appears as if the heart beat is, so to speak, maintained in virtue of the respiratory discharges. This condition can be produced artificially. For example, on raising the blood pressure in the head and in the thorax to a very high level the vagus tone may be increased to such an extent that the heart is almost stopped. If, however, the blood perfusing the head is exposed to a high tension of $CO_2$ and the respiration is thus maintained at a high frequency the heart begins to beat synchronously with each inspiratory act. As soon as the respiration is stopped by diminishing the $CO_2$ tension in the blood or by inflation of the lungs, the heart is no more driven by the activity of the respiratory centre and it assumes at once a remarkably slow rate, fig. 1, B, Plate 9.

The central arrhythmia is chiefly based on a diminution of the vagus tone during the periods of inspiration. It has been shown by Adrian and Buytendijk (1931) to what extent the state of the respiratory centre affects the whole structure of the medulla, the action currents being propagated to parts which are at a considerable distance away from the active centre. This observation lends support to the old theory of irradiation of impulses from the respiratory to the cardio-inhibitory centre.

Adrian, Bronk, and Phillips (1932), and recently Bronk (1935), observed volleys of action currents in the sympathetic fibres, which were synchronous with inspiration. It is not, however, easy to demonstrate the participation of the sympathetic fibres in the respiratory arrhythmia. Under normal conditions of quiet respiration the central arrhythmia usually becomes accentuated and not diminished after the extirpation of the thoracic sympathetic chain, fig. 2, A and B. As in the reflex arrhythmia, the periods of the cardiac slowing become more conspicuous on account of the absence of the sympathetic tone, while the periods of acceleration of the heart are affected but little. It is possible, in the innervated heart lung preparation, by slightly lowering the blood pressure perfusing the head of the animal, to keep the expiratory heart rate after

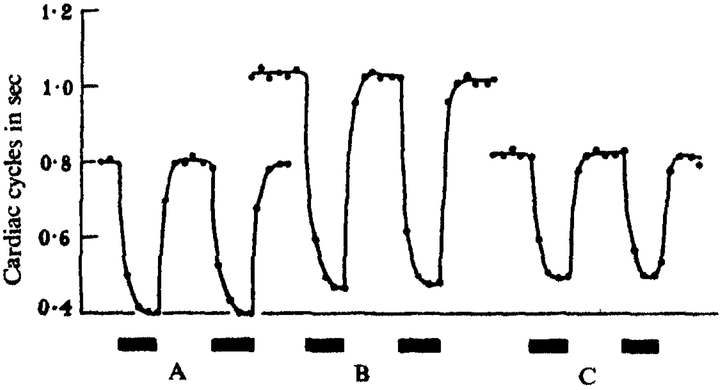


Fig. 2—To be read from left to right. Pulmonary vagi are cut. The bottom line represents the electrical deflexions of the phrenic nerve. The tracing shows the duration of the cardiac cycles in seconds. A, blood pressure 110 mm Hg; the sympathetic chain intact. B, blood pressure unchanged; after extirpation of the thoracic sympathetic chain. C, the same, but the blood pressure is dropped to 85 mm Hg so as to reduce the expiratory heart rate to what it had been before sympathectomy.

sympathectomy the same as it was before the section of the cardio-accelerator nerves. We generally found in such experiments that the central respiratory arrhythmia does not present a conspicuous difference under the two conditions. Only in a few experiments in which the respiration was increased in strength as a result of a mild asphyxiation of the animal could a certain difference before and after sympathectomy be noticed. Under these conditions the arrhythmia was more conspicuous before the extirpation of the sympathetic chain, fig. 2, A and C.

It would seem that the simplest way to demonstrate the participation of the accelerator fibres in the central respiratory arrhythmia would be to determine whether the arrhythmia persists after section of the cervical vagi. This leads, however, to such an acceleration of the heart that it is difficult to notice a difference between the inspiratory and expiratory heart rate. In fact, after having carefully measured a great number of records, we believe that the heart beats, under these conditions, at a uniform rate; at least, the occasional variations in the length of different cardiac cycles could not be attributed to the respiratory act. Nevertheless, by using the following technique it became possible to show that a certain tendency to arrhythmia is potentially present although it is masked by the extremely rapid heart rate following vagotomy. It is possible after section of the vagi in the neck, to keep the heart at a constant slow rate by a continuous excitation of the peripheral end of one of the vagi. If an induction coil is used, the experiment usually presents the difficulty that the artificial vagus tone does not remain constant. The heart slows at first more than necessary but, after a while, it begins to accelerate. This difficulty can be avoided if condenser discharges and non-polarizable electrodes are employed. It is easy to select a frequency and amplitude of the discharges which would slow down the heart rate for long periods of time and to any desired extent. Usually we selected such stimuli which reduced the heart beat to the rate it had during the periods of expiration before the section of the cervical vagi. It can be assumed that in the normal as well as in the artificial condition of the vagus tone the amount of acetylcholine liberated at the vagus nerve endings is the same, except that with the artificial tone the production of acetylcholine would proceed at a constant rate while under normal conditions it would vary in accordance with the state of the respiratory centre. If the sympathetic accelerator nerves become active during the phase of inspiration they should antagonize the artificially produced constant vagus tone. This actually occurred, as we found in a few experiments. During strong inspirations the heart rate was somewhat faster than during expirations. The effect completely disappears after extirpation of the thoracic sympathetic chain, fig. 3, Plate 9.

The respiratory arrhythmia of the central origin must be regarded, as the result of the above experiments, as based on the reciprocal co-operation of inhibitory and accelerator cardiac nerves. It will be remembered that we failed to demonstrate the participation of the sympathetic system in the reflex arrhythmia. It is, however, possible that we did not strike the best conditions in which it can be demonstrated. Experiments which were performed in Pavlov's laboratory by Nikiforovsky (1910) were interpreted as showing that the reflex acceleration of the heart is almost entirely due to the sympathetic accelerator mechanism. The conditions of these experiments were not, however, sufficiently well controlled. It is of interest, in relation to the above experiments, that Heymans and

Samaan (1934) find in surviving sympathectomized animals that the respiratory arrhythmia is not greatly changed, but that section of the cervical vagi abolishes it completely. These observations support our conclusions. Heymans and Samaan also find that the respiratory arrhythmia which was shown by H. E. Hering to disappear after the section of the vaso-sensory nerves, reappears during sleep of the animal (1934). Apparently the vagus tone returns in spite of the denervation of the carotid sinus and of the aorta.

## THE CORRELATION BETWEEN THE CENTRAL AND THE REFLEX MECHANISMS OF THE RESPIRATORY ARRHYTHMIA

In describing the reflex and the central mechanisms of the arrhythmia, we emphasized the necessity of studying them separately in conditions in which only one or the other of these mechanisms would be present. In order to study the reflex mechanism the activity of the respiratory centre was abolished by supplying the head with blood of a low $CO_2$ tension. In order to study the central arrhythmia the pulmonary reflexes were abolished by section of the vagi above the lungs. The investigation of either mechanism in presence of the other is rendered extremely difficult on account of the fact that the lungs, besides the effect which they exert on the heart rate, also have a most powerful influence on the activity of the respiratory centre. Inflation of the lungs causes an arrest of respiration, deflation of the lungs causes an acceleration of the respiration. It is immaterial for the present problem whether these effects are due, as suggested by Adrian (1933), to a single act of inhibitory fibres which are stimulated only during the periods of inflation or, as is more likely, to the action of two sets of nerve fibres one predominating during the expansion of the lungs and the other during their deflation (Hammouda and Wilson, 1935). Whatever the explanation may be, the result is the same, the collapse of the lungs greatly and immediately accelerates the respiration. The cardio-inhibitory centre, therefore, is, on the one hand, influenced by the deflated state of the lungs and, on the other, by the conspicuous increase in the activity of the respiratory centre. The reverse takes place during inflation of the lungs. It should be expected that had there been no cardio-accelerator impulses originating within the lungs the heart rate would slow down during each inflation as the result of the inhibition of the respiratory centre. In other words, under normal conditions the inflation of the lungs, while directly inhibiting the vagus centre, produces indirectly an excitation of it through the respiratory centre. Also the deflation of the lungs, while removing the directly acting cardio-accelerator impulses arising in the lungs, leads indirectly to a decrease of the tone of the vagus centre through stimulating the respiration.

The effects which the lungs exert on respiration are much more powerful and stable than the effects which they directly exert on the heart rate. Even when the lungs are in an advanced pathological state, as for instance in pulmonary oedema, inflation still continues to arrest, and deflation to accelerate, the respiration. The normal reflex cardiac effect originating in the lungs will be by that time absent. Once the cardio-accelerator pulmonary influence is abolished, inflation of the lungs leads to an indirect slowing of the heart rate through arresting the respiration. This condition has been observed by us in several experiments. It can be produced artificially. If the lungs are expanded with a considerable force and their expansion is maintained for a number of minutes the accelerator effect of inflation usually disappears for the rest of the experiment. The influence of the lungs on the respiration remains, however, unaltered. If the vagus tone is still present inflation is now accompanied by a slowing of the average heart rate per minute instead of an acceleration, while deflation causes a considerable acceleration instead of the usual slowing. It can easily be shown that these effects have nothing to do with the direct influence which inflation and deflation respectively exert on the vagus centre. As soon as the $CO_2$ tension of the blood perfusing the animal's head is reduced and the respiration becomes abolished, inflations and deflations cease to affect the heart rate. For example, in one experiment, after having abolished the pulmonary reflexes by means of a temporary over-expansion of the lungs, their inflation caused a slowing of the average heart beat from 120 to 82 beats per minute. On diminishing the $CO_2$ supply to the head the respiration stopped, the heart rate established itself at 86 beats per minute, and was unaltered by pulmonary inflations. In some experiments the effect was still more conspicuous. It is likely that the cardio-inhibitory effect of the insufflation of various irritant vapours into the lungs may, in many cases, also be caused indirectly through the arrest of the respiratory activity and not through a direct cardio-pulmonary reflex.

The complicated interrelations existing between the pulmonary ventilation, the respiration, and the heart rate show the difficulties with which the investigator has to deal. If the blood pressure is not controlled as would occur in most experiments on the whole animal, then the picture becomes so complicated that it is hardly surprising that so many observers disagree with each other.

### *The Relative Magnitude of the Central and Reflex Effects*

It is well known that in experiments with artificial ventilation of the lungs the respiratory discharges are interposed between the periods of pulmonary inflation. Each period of inflation of the lungs is followed by an act of inspiration. It would be expected, on the basis of the foregoing discussion, that in conditions of artificial ventilation in the lungs in the presence of a respiratory activity two phases of respiratory arrhythmia should be in evidence; one of central origin coinciding with the inspiration, the other of a reflex origin coinciding with the pulmonary inflation. This double respiratory arrhythmia can be extremely easily demonstrated. Fig. 4 shows two superimposed tracings, one taken from a record obtained during administration of a mixture of 4·2% $CO_2$ in air to the heart lung preparation perfusing the head, the other during administration of pure air. In the first case the respiratory movements of the larynx alternated with the inflations of the lungs, in the second the respiration was absent. One can see how the slowing of the heart, which develops, in absence of respiration, between the period of inflation of the lungs, is cut short and replaced by a second period of acceleration as soon as the respiratory activity starts. It is not surprising that it is so difficult to demonstrate

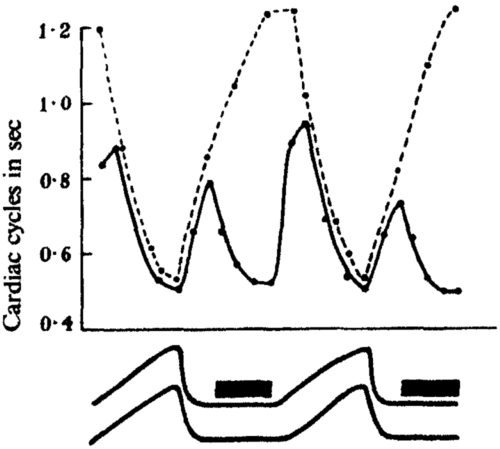


FIG. 4—To be read from left to right. The bottom tracing shows the pulmonary inflations, the respiration is absent, the head being perfused with blood of a low $CO_2$ tension. The corresponding single respiratory arrhythmia is shown by the dotted line. The second tracing from below represents the inflations of the lungs and the respirations as recorded by the largyngeal movements, the head being perfused with blood exposed to 4·2% $CO_2$. The corresponding double respiratory arrhythmia is shown by the continuous line.

the respiratory arrhythmia in animals under artificial ventilation. The vagus centre is constantly inhibited, once by the inspiration and once by the inflation. It becomes, however, easy to demonstrate the central arryhthmia after the section of the pulmonary vagi. The slowing of the heart which develops between the respirations is not antagonized any longer by an acceleration of pulmonary origin, fig. 5, Plate 10. Similarly, it is easy to demonstrate the reflex respiratory arrhythmia in animals the respiratory activity of which is in abeyance. The slowing of the heart which develops between each inflation of the lungs is no longer antagonized by the inspiratory activity intervening between the inflations.

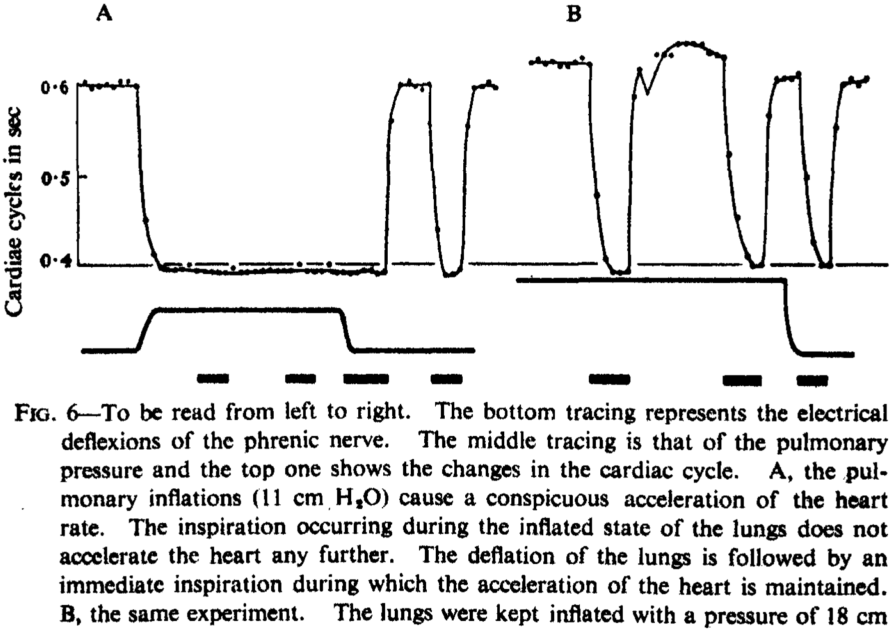


Fig. 6—To be read from left to right. The bottom tracing represents the electrical deflexions of the phrenic nerve. The middle tracing is that of the pulmonary pressure and the top one shows the changes in the cardiac cycle. A, the pulmonary inflations (11 cm $H_2O$) cause a conspicuous acceleration of the heart rate. The inspiration occurring during the inflated state of the lungs does not accelerate the heart any further. The deflation of the lungs is followed by an immediate inspiration during which the acceleration of the heart is maintained. B, the same experiment. The lungs were kept inflated with a pressure of 18 cm of water until the " secondary slowing " became completely established. Inspirations occurring during the secondary slowing caused an acceleration of the heart which was of the same order as in A.

In some animals the reflex arrhythmia is more pronounced than the central one, in others it is the reverse. Usually the extent of the acceleration of the heart observed during the inflation is the same as during the inspiration. This can easily be made evident when an inspiration immediately follows a rapid deflation of the lungs. In this case the slowing due to the deflation has not yet had time to develop when the heart beat is again accelerated by the inspiratory activity, fig. 6, A. The maximum acceleration of the heart is the same whether the inspiration is short or prolonged, the only difference is in the duration of the period of acceleration.

In many experiments we tried to evoke an inspiratory act during the inflated state of the lungs in order to find whether there is a summation of the two arrhythmias. When the head of the animal is perfused with blood containing a very high concentration of $CO_2$ the inflation of the lungs may lead only to a temporary arrest of the respiration. The respiratory centre continues to discharge in spite of the inflated state of the lungs. Thus the central respiratory arrhythmia should, so to speak, become superimposed upon the effect of the sensory impulses arising in the lungs. When the cardio-accelerator pulmonary impulses are still present inspiration does not, as a rule, cause a further quickening of the heart rate, fig. 6, A. When, however, the accelerator impulses have been weakened and the inflation of the lungs has led to a " secondary slowing," an inspiration of the animal is always accompanied by a considerable acceleration of the heart, fig. 6, B. In the majority of our observations, the acceleration was as conspicuous as that observed before the development of the secondary slowing. It seems, therefore, that no real summation between the two arrhythmias exists. One, in the majority of cases, acts as strongly as both together.

The above experiments also give additional support to the conclusions reached in the previous paper, namely, that the secondary slowing is an expression of the phenomenon of adaptation of the nerve endings in the lungs and is not due to an increase in the excitability of the cardio-inhibitory centre. It would be expected, if the latter were true, that the respiratory acceleration of the heart of central origin would be less conspicuous when an inspiration occurs during the secondary slowing since it would then fall upon a less responsive vagus centre. It is seen, however, that the respiratory acceleration of the heart is not changed during the secondary slowing. The respiratory arrhythmia becomes relatively increased because of the slower rate which the heart has in between the periods of inspiration.

## GENERAL INTERPRETATION OF THE EXPERIMENTS AND CONCLUSIONS

As a general conclusion arrived at as a result of all our experiments upon the respiratory cardiac arrhythmia, we should like to present the diagram shown in fig. 7. The vagus centre stands under two inhibitory influences which directly act upon it; one arises in the lungs and is especially strong during their inflation, the other arises in the respiratory centre. In addition to this the tone of the vagus centre is affected by the

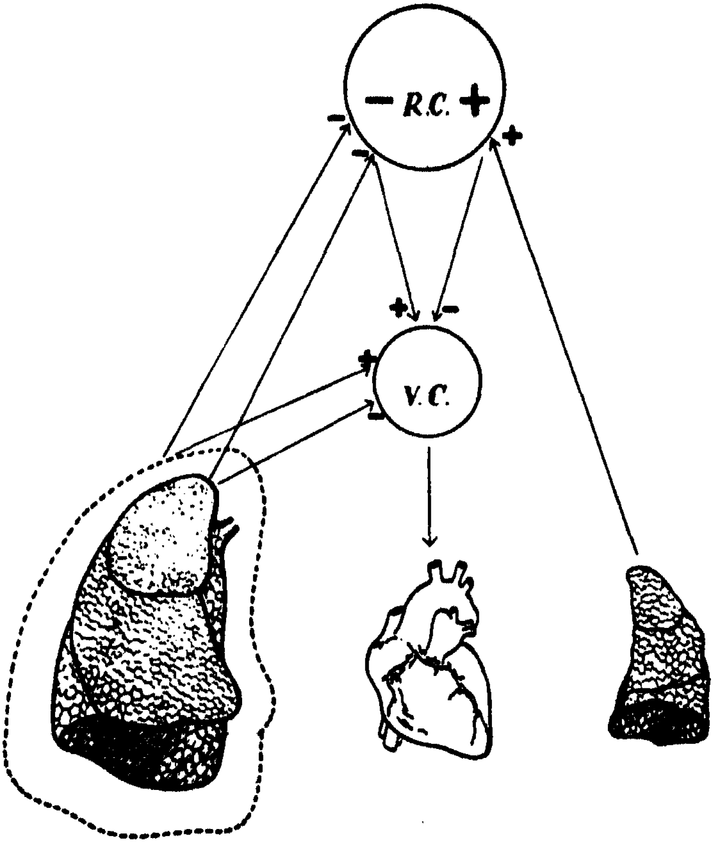


FIG. 7—A diagrammatic representation of the interrelations between the respiratory act and the heart rate. R.C., the respiratory centre; V.C., the vagus centre; + denotes excitation; − denotes inhibition. On the right-hand side the collapse of the lungs is shown to induce an inhibition of the vagus centre brought about indirectly through exciting the respiratory centre. On the left-hand side is shown an inflated lung which causes a direct inhibition of the vagus centre and simultaneously an indirect excitation of it brought about by inhibiting the respiratory centre. The dotted contour of the lungs illustrates an over-expanded lung which causes the opposite effect, namely, a direct excitation of the vagus centre accompanied by an inhibition of the respiratory centre. These latter effects have nothing to do with the respiratory arrhythmia. The part played by the sympathetic nervous system is described in the text but omitted from this diagram.

lungs in an indirect way through the Hering-Breuer reflex. Thus each inflation affects the vagus centre in two opposite ways. On the one hand, by a direct reflex it induces an inhibition of the vagus centre and, on the other hand, simultaneously but indirectly it antagonizes this inhibition by cutting short the activity of the respiratory centre. Conversely, a deflation of the lungs diminishes the direct reflex inhibition of the vagus centre by cutting short the afferent impulses reaching it from the lungs, but at the same time it indirectly antagonizes this effect by stimulating the activity of the respiratory centre. The pulmonary reflexes are in every respect more delicate and more difficult to observe than the central changes in the heart rate. Very severe inflations of the lungs evoke stimuli which have no relation to the respiratory arrhythmia proper. Under these conditions the vagus centre is greatly stimulated so that the heart may be brought to a standstill (von Saalfeld, 1932, 1933).

The picture presented by the respiratory arrhythmia is an extremely complicated one. The mechanisms described here can, by superimposition or by interference, give all sorts of complex effects. We are so much aware of the absolute necessity of a perfectly rigid control of all experiments on respiratory arrhythmia that we should like to warn those who intend to investigate it not to start with experiments on the whole animal but at first to make their observations on standard preparations similar to those used by us. The conditions affecting the respiratory arrhythmia are so numerous that it becomes difficult to control them in experiments on the whole animal and easy to arrive at conclusions which may be completely erroneous.

## REFERENCES.

Adrian, E. D. (1933). ' J. Physiol.,' vol. **79**, p. 332.

Adrian, E. D., Bronk, D. W., and Phillips, G. (1932). ' J. Physiol.,' vol. **74**, p. 115.

Adrian, E. D., and Buytendijk, F. J. J. (1931). ' J. Physiol.,' vol. **71**, p. 121.

Bronk, D. W. (1935). ' Proceedings of XVth Int. Physiol. Congr.,' Russia.

Frédéricq, L. (1882). ' Arch. Biol. Paris,' vol. **8**, p. 55.

Hammouda, M., and Wilson, W. H. (1935). ' J. Physiol.,' vol. **85**, p. 62.

Hering, E. (1871). ' S.B. Wien, Akad. Wiss.,' vol. **64** (2), p. 333.

Heymans, C. (1929). ' Ergebn. Physiol.,' vol. **28**, p. 292.

Heymans, C., and Samaan, A. (1934). ' C.R. Soc. Biol. Paris,' vol. **140**, p. 1383.

Heymans, C., Samaan, A., and Bouckaert, J. J. (1934). ' Arch. Int. Pharmacodyn.,' vol. **48**, p. 457.

Heymans, J. F., and Heymans, C. (1928). ' Arch. Sci. Biol.,' vol. **12**, p. 153.

Nikiforovsky, P. M. (1910). ' Proc. Military Acad. St. Petersburg,' p. 221.

Saalfeld, E. von (1932). ' Pflüger's Arch.,' vol. **281**, p. 33.

—— (1933). ' Pflüger's Arch.,' vol. **281**, p. 724.

Snyder, C. D. (1915). ' Amer. J. Physiol.,' vol. 37, p. 104.

Traube, J. (1865). ' Zbl. med. Wiss.,' No. 56.

## DESCRIPTION OF PLATES

### PLATE 9

FIG. 1—To be read from right to left. Time in 0·04 second. The laryngeal movements corresponding to inspiration and the electro-cardiogram are recorded simultaneously. The figures on the records show the duration of the respective cardiac cycles. A, both vagi are cut immediately above the lungs. The blood perfusing the head of the animal is exposed to 3·2% $CO_2$. The respiration is vigorous and slow. The heart rate abruptly accelerates during inspiration to more than double. At the end of inspiration it returns within one heart beat to the slow expiratory rate. Mean blood pressure 95 mm Hg. B, The blood pressure is 170 mm Hg and the $CO_2$ to which the blood perfusing the head is exposed is 8·6%. The pulmonary vagi are not cut. The lungs are arrested in a state of deflation. The respiration is fast on account of the high $CO_2$ tension. The heart rate is synchronous with each inspiration. On inflating the lungs the respiration was immediately stopped and this was accompanied by an extreme slowing of the heart rate, the corresponding cardiac cycles being over 5 seconds each.

FIG. 3—To be read from right to left. Both vagi are cut in the neck. The peripheral end of the right vagus is stimulated with condenser discharges at the rate of 150 per second. During inspiration the heart rate shows a definite acceleration. This effect disappeared after extirpation of the thoracic sympathetic chain.

### PLATE 10

FIG. 5—To be read from right to left. The bottom tracing in each record shows the inflations of the lungs, maximum inflation was 11 cm of water. The second tracing is that of the laryngeal movements and the third is that of the electro-cardiogram. Time in 0·04 second. A, the respiratory arrhythmia is almost completely masked by the alternating reflex effect of pulmonary inflation and of central effect of the respiration. The pulmonary vagi are intact. B, the same experiments a few minutes after section of both vagi immediately above the lungs. The respiratory arrhythmia becomes very conspicuous. A segment of the tracing was selected in which the inflation happened to intervene between the respirations.

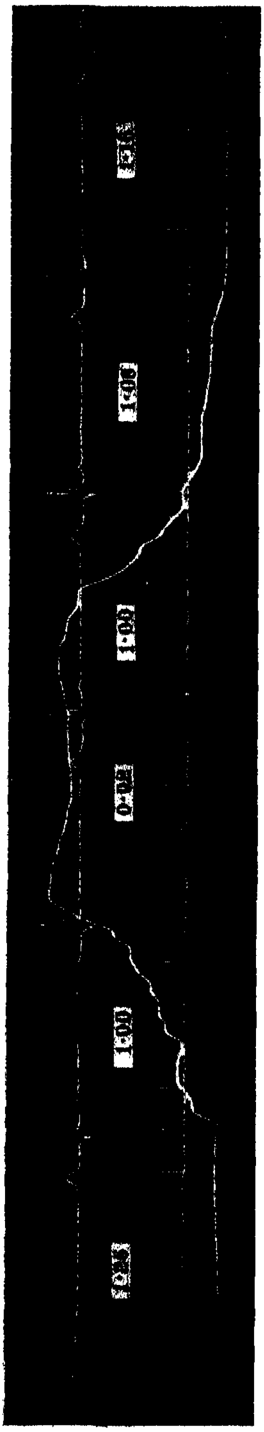

1·00
0·98
1·03
1·06

FIG. 3.

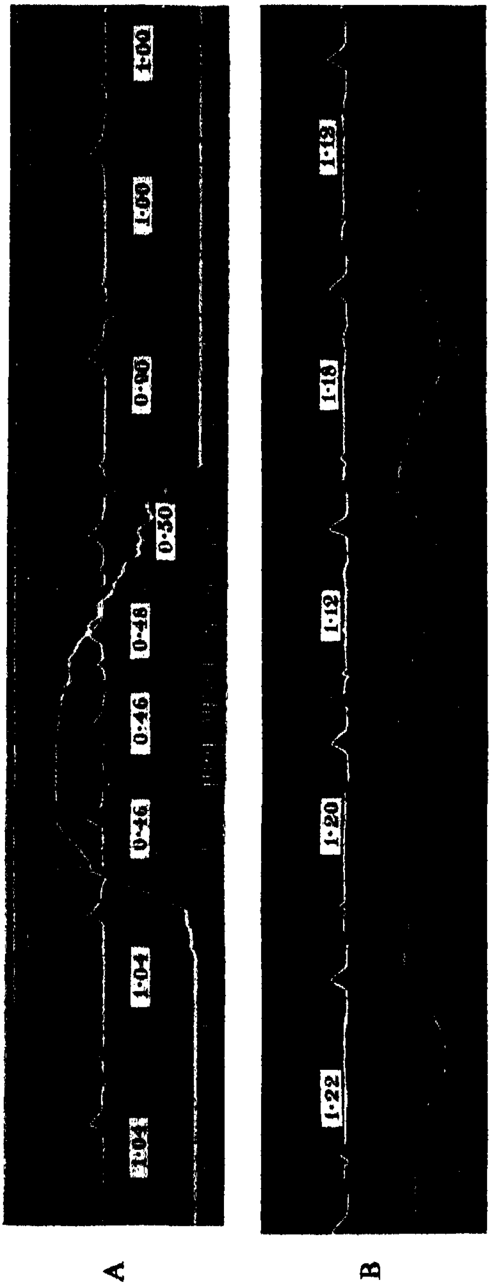

1·02
1·04
0·46
0·46
0·48
0·50
0·96
1·00
1·00
1·22
1·20
1·12
1·18
1·13
A
B

FIG. 1.

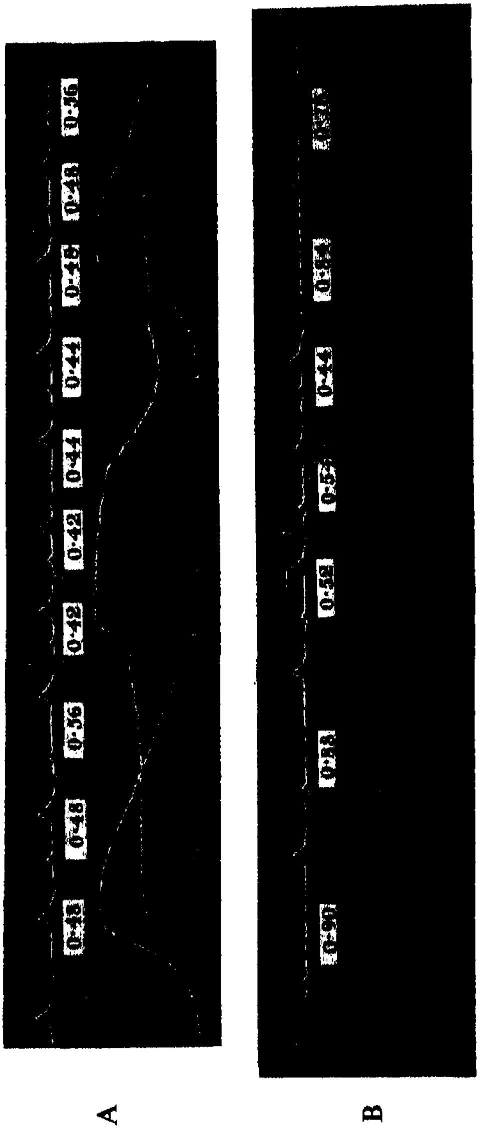


FIG. 5.

537 · 363

# Studies in Microcataphoresis
## I—Technique

By H. C. Brown and J. C. Broom, Wellcome Bureau of Scientific Research, London

(*Communicated by C. M. Wenyon, F.R.S.—Received August* 19, 1935)

A critical survey of the literature which has now accumulated on the subject of microcataphoresis reveals an apparent omission and disregard of certain essential details of technique without which concordant results are quite impossible.

In many instances also, conclusions appear to have been drawn from an insufficient number of observations and there is no evidence of any attempts to define the margin of error in the published figures. In certain cases the observers cannot agree regarding even the sign of the charge carried by the particles under observation. This may be due to lack of a full appreciation of the conditions present in cells differing in construction. Authoritative work such as that of Abramson, Northrop, Mudd, Kross, and Zuelzer, Dummett and Bowden, Henry and others indicates the difficulties which are likely to be encountered, but as far as we know no detailed description of the complete technique has ever been published.

The present paper is an attempt to describe fully the technique we have finally adopted for a microcataphoresis cell.

Although we have used one type of cell throughout, we consider that our findings are applicable to other types of microcataphoresis cell. The cell is a modification of that devised by Northrop (1922), though we have found it advisable to introduce certain alterations into the pattern of the cell originally described by us (Brown and Broom, 1929).

In fig. 1 we have shown the structure of the cell. The upper and lower plates are of optical glass. The small endpieces R and S can be removed, so allowing the trough of the cell to be cleaned. The zinc electrodes are connected to the source of supply of current. We have found that batteries of large capacity are more satisfactory than a mains supply especially when an electrostatic voltmeter is used.

We originally determined the potential gradient by the method of specific conductivity, but found that the correction necessary for the varying cross-sections of the different parts of the cell was both laborious and uncertain. To overcome this difficulty, platinum electrodes were

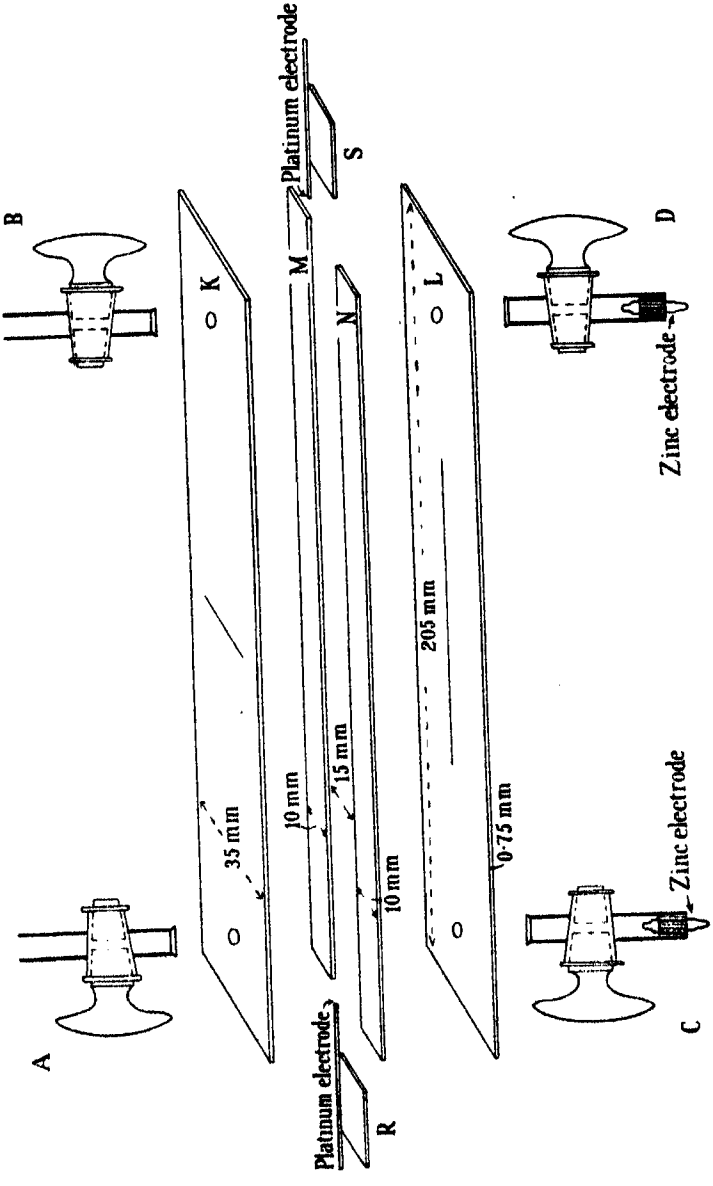

B
Platinum electrode
S
K
M
N
L
D
Zinc electrode
205 mm
35 mm
10 mm
15 mm
10 mm
0·75 mm
Zinc electrode
A
C
Platinum electrode
R

FIG. 1.

inserted at the ends of the trough. Between these two points the cross-section is constant, and hence the voltage drop in the cell proper can be determined directly by connecting the electrodes to an electrostatic voltmeter.

### MEASUREMENT OF THE DEPTH OF THE CELL

In order that the depth of the cell can be determined with accuracy, extremely fine lines are scratched on the under surface of the top plate and the upper surface of the lower plate. These must be sufficiently fine not to interfere with the dark field illumination. A ring of 1·5 mm diameter is scratched upon the centre of the upper plate and all observations are made through the centre of this circle.

The apparent depth of the cell must be determined when it has been filled with a sample of the actual suspension under observation because the depth as measured varies with the refractive index of the solution.

The depth is estimated as follows: with the cell filled and clamped in position on the stage of the microscope the milled head of the fine adjustment is set to zero at the bottom of its excursion. The lines on the upper surface of the lower plate are focussed approximately with the coarse adjustment. Critical focussing on these lines is subsequently made with the fine adjustment, care being taken that the final movement of the objective is always downwards. This precaution is necessary to avoid errors due to accommodation. These lines having been focussed with the utmost precision possible, and the reading of the fine adjustment noted, the objective is racked up by the fine adjustment until the lines on the lower surface of the upper plate are in critical definition, the final focussing being downwards as before.

The depth of the cell is expressed in terms of the graduations of the fine adjustment.

### THE MICROSCOPE

From the above it is obviously of the utmost importance that the mechanics of the microscope should ensure the movement of the objective to be a linear function of the number of revolutions of the fine adjustment.

Kross and Zuelzer (1932) have drawn attention to the fact that this is not true of all microscopes. We have examined a large number of microscopes both of British and of foreign manufacture and have found the majority unsatisfactory because the fine adjustment depends on the action of a cam. It might seem possible to find some portion of the excursion where the relation is almost linear as checked by micrometer readings, but for critical work this has not proved satisfactory.

Apart from obtaining a microscope specially made for this purpose, we have found that the large types of microscopes manufactured by Emil Busch Optical Co. Ltd.* most nearly approximate our requirements. In these microscopes the fine adjustment consists of a steel roller bearing on an inclined plane, thus giving the required relationship.

We have tested the suitability of the fine adjustment of the microscope in the following way: microscope slides of the same refractive index but of varying thicknesses are taken and a 1·5 mm circle is scratched on each. Fine marks above and below the slide, within the circle, are focussed by means of the fine adjustment and the readings recorded. These measurements should bear a constant relation to the actual thickness as found by a micrometer screw gauge.

Only with a Busch microscope, specially adjusted for this work by a competent mechanic, have we been able to obtain absolutely satisfactory results.

It is necessary to repeat this test at frequent intervals to be certain that no wear has developed in the fine adjustment, because even a minute degree of " backlash " will completely invalidate the results.

## THE OPTICAL SYSTEM

It is preferable, as Kross and Zuelzer also note, to use an Erlich's eyepiece when making preliminary measurements. We have used a 10 × ocular and 6 mm objective fitted with a funnel stop for dark field illumination, and also a 25 × ocular with a 16 mm objective. Inside each eyepiece is placed a circular coverslip engraved with a measured square which covers only the flat portion of the field.

## ILLUMINATION

As this cell is intended for dark field illumination it is advisable to employ either a " Pointolite " or a mercury vapour lamp. Owing to the heat given off from these lamps it is absolutely essential to interpose a large glass screen between the lamp and the microscope as well as a parallel sided cell containing a saturated solution of alum. Without these we have repeatedly demonstrated that the velocity of particles in the lower portions of the cell far exceeds that in the upper layers, owing to differences of temperature in the two positions.

---

* We desire to express our thanks to Mr. A. G. Frewin, Director of the London branch of this firm, for his advice and for helping us to overcome our difficulties in this matter.

## FILLING THE CELL

The zinc electrodes, passed through a small piece of rubber tubing, are placed securely in position. A capillary pipette containing a saturated solution of sulphate of zinc is inserted through the upper tap into the lower compartment and the latter is completely filled up to and including the channel in the tap. The lower taps are closed and the cell is thoroughly flushed, first with tap water, then with distilled water. Finally the cell is washed with the suspending fluid and left for sufficient time to allow adsorption equilibrium to be established between the walls of the cell and the medium. For a solution of gelatin, Dummett and Bowden (1933) allowed interaction to take place for 2 hours. When an electrolyte solution is the suspending medium, half an hour has appeared to be sufficient in our experience.

We have found that some samples of " pure " zinc sulphate show an intensely acid reaction, so that polarization occurs when a current is passed through the cell. This can be obviated by recrystallization of the salt.

## THEORETICAL CONSIDERATIONS

It will be necessary to consider first whether the observation of the cataphoretic behaviour of particles in such a cell will give a true representation of the sign and degree of electric charge of the particles.

In accordance with Coehn's (1898) rule, the glass walls of the cell are charged negatively with regard to the water because of the higher dielectric constant of the water. If a potential is applied to the two ends of the cell, the water in the vicinity of the walls (which is positively charged) will move towards the cathode. As the cell is closed, there can be no transport of water as a whole and there must therefore be a return flow towards the anode down the centre of the cell.

Since the movement of the fluid at the walls is in the opposite direction to the movement at the centre, there must be positions at which the fluid is stationary.

## DETERMINATION OF STATIONARY LEVELS

Ellis (1911), who used a rectangular cell, studied the migration rates of particles at different levels. He found that the apparent migration rate of the particles at any depth was a parabolic function of the distance below the coverglass. From graphical integration of his results he concluded that the true velocity V could be obtained from the empirical formula $V = 0{\cdot}361\ V_0 + 0{\cdot}639\ V_{\frac{1}{2}}$, where $V_0$ and $V_{\frac{1}{2}}$ are the apparent velocities immediately under the coverslip and in the middle of the cell.

Smoluchowski (1921) investigated this question from a theoretical standpoint. He showed that the formula suggested by Ellis should actually be

$$V = 0{\cdot}333\, V_0 + 0{\cdot}667\, V_{\frac{1}{2}},$$

and that the dependence of the apparent velocity on the depth would be represented by a parabola. He calculated that the velocity of the endosmotic streaming, that is the movement of the fluid at any distance X below the upper wall, can be obtained from the formula

$$v_x = v_0 \left[ 1 - 6 \left\{ \frac{x}{d} - \left( \frac{x}{d} \right)^2 \right\} \right],$$

where

$v_x$ = the velocity of the fluid at the point $x$,

$v_0$ = the velocity of the fluid immediately under the wall,

$d$ = the total depth of the cell.

It is obvious that the stationary layer is at a level where $v_x = 0$; that is to say a depth at which there is no movement of the fluid. The values of $x$ are obtained by equating $v_x$ to 0 in the above expression, *i.e.*,

$$0 = v_0 \left[ 1 - 6 \left\{ \frac{x}{d} - \left( \frac{x}{d} \right)^2 \right\} \right]$$

and

$$x_{v=0} = d\left(\tfrac{1}{2} \pm \sqrt{\tfrac{1}{12}}\right),$$

*i.e.*, the stationary layers are at a distance $d/2\sqrt{3}$ above and below the middle of the cell.

The true velocity of a migrating particle can therefore be obtained directly in the stationary layer.

Kross and Zuelzer point out that, in addition to taking the velocity at the stationary layer, it is advisable to verify it by measurements at other levels. From Smoluchowski's formula these observers calculated the corrections to be applied to observations made at different levels in order to obtain the true velocity. These are shown in Table I.

In their experience the most satisfactory results are obtained when measurements at $d/10$, $d/8$, and $d/6$ are associated with those at $d/2$. Powis (1914–15) had previously used a similar formula,

$$V = \frac{V_{\frac{1}{2}} + 0{\cdot}565\, V_0}{1{\cdot}565},$$

to find the true velocity.

Komagata (1933) points out that Smoluchowski's calculations are based on the assumption that the cell is so wide that the side walls have no effect on the endosmotic flow in the cell. In narrow cells this effect must be taken into consideration, so Komagata has modified Smoluchowski's equation to make allowance for the width. He finds that when the vessel is of the totally enclosed type the satationary layers are given by the equation

$$x_{v=0} = b \pm b\sqrt{1/3\left(1 + \frac{384}{\pi^5 K}\right)}.$$

where $2b$ = the depth; $K = \frac{\text{the width}}{\text{the depth}}$.

TABLE I

| Depths observed | True electrophoretic velocity |
|---|---|
| 0 and $d/2$ | $V = 0{\cdot}333\,V_0 + 0{\cdot}667\,V_{1/2}$ |
| $d/10$ and $d/2$ | $V = 0{\cdot}52\,V_{1/10} + 0{\cdot}48\,V_{1/2}$ |
| $d/8$ and $d/2$ | $V = 0{\cdot}593\,V_{1/8} + 0{\cdot}407\,V_{1/2}$ |
| $d/6$ and $d/2$ | $V = 0{\cdot}75\,V_{1/6} + 0{\cdot}25\,V_{1/2}$ |
| $d/4$ and $d/2$ | $V = 1{\cdot}333\,V_{1/4} - 0{\cdot}333\,V_{1/2}$ |

Reproduced from Kross and Zuelzer (1932).

Abramson (1934) has shown that this correction is unnecessary in cells whose width is at least twenty times the depth. We have confirmed this by comparing the effect of this correction in narrow and wide cells.

TABLE II—SHOWING HOW THE VELOCITIES IN THE SMOLUCHOWSKI AND KOMAGATA STATIONARY LAYERS APPROXIMATE AS THE WIDTH/DEPTH RATIO INCREASES

| $\frac{\text{Width of cell}}{\text{Depth}}$ | Velocity according to | |
|---|---|---|
| | Smoluchowski | Komagata |
| K = 5·8 | 4·89 | 5·42 |
| K = 7·2 | 4·60 | 5·04 |
| K = 11·0 | 4·81 | 5·04 |
| K = 17·0 | 5·02 | 5·19 |
| K = 19·8 | 4·83 | 4·89 |
| K = 25·0 | 5·26 | 5·26 |

Table II shows that as the ratio of width to depth of the cell increases the difference between the velocities at the Komagata and Smoluchowski "stationary" layers decreases. These results are the means of 20 observations in each case.

These particular observations were made on different occasions and on sols prepared under different conditions; this explains why the velocities in the Komagata layers are not constant. It might be mentioned that all conditions must be standardized meticulously in order to prepare on different occasions mastic sols which will behave identically. We have used these standard conditions only when proving the repeatability of results in different cells from day to day. We first tried to use a sol which had aged at room temperature for 6 months. This sol, however, was unsatisfactory because individual particles showed very marked differences in their migration rates, thus giving a coefficient of variation much too high for our purpose.

TABLE III—SHOWING THE MANNER IN WHICH THE DIFFERENT METHODS OF CALCULATING THE VELOCITY OF A PARTICLE COINCIDE, IN A NARROW CELL, WITH THE VELOCITY OBSERVED IN THE SMOLUCHOWSKI RATHER THAN THE KOMAGATA STATIONARY LAYER

| Width/depth = K of cell | Velocity calculated from velocities observed at | | | | Velocity from integration of curve | Velocity observed directly in | |
|---|---|---|---|---|---|---|---|
| | $d/10$ and $d/2$ | $d/8$ and $d/2$ | $d/6$ and $d/2$ | $d/4$ and $d/2$ | | Smoluchowski layer | Komagata layer |
| 5·8 | 5·56 | 5·38 | 5·47 | 5·29 | 5·46 | 5·41 | **4·89** |
| 6·0 | 5·16 | 5·09 | 5·11 | 5·05 | 5·08 | 5·07 | **4·65** |
| 6·2 | 5·05 | 4·96 | 4·94 | 4·91 | 4·92 | 4·93 | **4·46** |
| 6·7 | 4·84 | 4·73 | 4·72 | 4·62 | 4·76 | 4·75 | **4·08** |
| 19·8 | 5·19 | 5·14 | 5·10 | 5·12 | 5·12 | 5·08 | **4·96** |
| 24·8 | 5·39 | 5·34 | 5·36 | 5·45 | 5·35 | 5·26 | **5·26** |

In this connexion we have found, for narrow cells in which the ratio of the width to depth is of the order of 10 or less, that the velocities obtained by integration of the parabola, by observations at different levels and directly in Smoluchowski's " stationary " layer approximately coincide, but all differ markedly from the velocity in the Komagata layer. This would explain why, when using cells of the same dimensions, the results of critical experimental work appeared to substantiate the validity of Smoluchowski's calculations. When, however, an attempt is made to correlate the results obtained in cells of very different widths and depths, discrepancies appear which can only be rectified by the application of Komagata's correction.

Table III shows the velocities as obtained by these different methods in a number of narrow cells. As before, the sols are not prepared under standard conditions but for each the " Komagata velocity " differs from all the others which are relatively consistent among themselves.

## PROOF THAT THE CELL IS SATISFACTORY

A series of experiments was undertaken as a critical test of the cell. These were designed to show (*a*) that the cell gives a symmetrical parabola of observed velocity throughout its depth as demanded by theory; (*b*) that the true velocity of a particle can be obtained from the cell; and (*c*) that repeatable results are possible.

Fig. 2 shows how closely the parabola calculated from Smoluchowski's equation fits the observations in a certain experiment. It is practically impossible to obtain the " observed " velocity of the particle immediately under the coverglass. The method adopted, therefore, was to fit a parabola, by the method of least squares, to the velocities observed at depths from 1/8 to 7/8 and extrapolate the velocity at depth 0. The velocities calculated from the equation to the fitted parabola and by Smoluchowski's equation are given together with the observed velocities in Table IV.

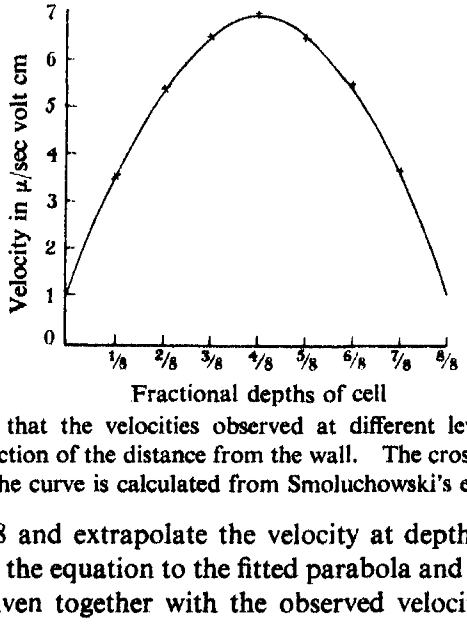


FIG. 2—Showing that the velocities observed at different levels of the cell are a parabolic function of the distance from the wall. The crosses show the velocities observed. The curve is calculated from Smoluchowski's equation.

TABLE IV—SHOWING THAT THE OBSERVED AND CALCULATED VELOCITIES COINCIDE

| Depth below coverglass $= x/d$ ............ | 0 | 1/8 | 2/8 | 3/8 | 4/8 | 5/8 | 6/8 | 7/8 | 8/8 |
|---|---|---|---|---|---|---|---|---|---|
| Velocity observed at depth $x/d$ ........ | — | 3·52 | 5·42 | 6·50 | 6·99 | 6·43 | 5·48 | 3·62 | — |
| Velocity calculated from fitted curve ........ | 1·04 | 3·58 | 5·40 | 6·50 | 6·96 | 6·52 | 5·44 | 3·64 | 1·10 |
| Velocity calculated from Smoluchowski's equation .............. | 1·04 | 3·47 | 5·40 | 6·47 | 6·91 | 6·47 | 5·40 | 3·47 | 1·04 |

Velocities are expressed in μ/sec volt cm.

## CORRELATION OF RESULTS OBTAINED WITH CELLS OF DIFFERENT DIMENSIONS

We have repeatedly shown that although the " Smoluchowski velocity " is the same in two or more cells of the same depth and width it is impossible to correlate the results in cells with different K ratios. Table V shows, however, that when Komagata's formula is applied identical results are obtained in cells widely differing in length, breadth, and depth. Each result is the mean of 40 observations; its standard error is also given.

TABLE V—SHOWING THAT THE VELOCITY OF A PARTICLE IS THE SAME IN THE " KOMAGATA STATIONARY LAYER " IN DIFFERENT CELLS

| Depth of cell in mm | $\frac{\text{Width}}{\text{Depth}} = \text{K}$ | Velocity in $\mu$/sec volt cm | Standard error |
|---|---|---|---|
| 0·95 | 6·0 | 5·18 | ±0·04 |
| 0·80 | 6·7 | 5·19 | ±0·05 |
| 0·83 | 17·4 | 5·30 | ±0·06 |
| 0·76 | 19·8 | 5·22 | ±0·04 |

Each result is the mean of 40 observations.

When using solutions with an electrolyte content of the order of 10 millimols per litre, we have found it preferable to use a cell with a K ratio between 6 and 7. This obviates any tendency to polarization of the electrodes from the passage of excessive current.

## REPEATABILITY OF RESULTS

To test this point a gum mastic sol was prepared under the following standard conditions. A stock solution of phosphate buffer $p_{\text{H}}$ 7·4 was made containing 89·34 millimols of electrolyte per litre. 50 cc of the stock solution were diluted to 500 cc with boiled, cooled, distilled water. To 50 cc of the diluted buffer were added 3 drops of a 10% solution of gum mastic in absolute alcohol. The same pipette was used on every occasion, and the drops allowed to fall into the buffer from a height of 10 cm, the buffer being gently agitated during the addition of the mastic. The mastic sol was filtered through paper to remove large particles.

The same cell was used throughout the test. One hundred observations were made in the stationary layer on each of five occasions. The means and standard errors of the means are given in Table VI.

These experiments show that the type of cell described gives a symmetrical parabola of apparent velocity at various depths; that the true velocity can be obtained in the stationary layer; and that accurate and repeatable results are possible.

It would appear, therefore, that such a cell is satisfactory for the study of microcataphoresis, and also that one is justified in assuming that the true velocity of a particle can be observed directly in the calculated stationary layer.

With most biological material, such as bacteria, a greater variation occurs. For example, the coefficient of variation of the mean of a series of 100 observations for a gum mastic sol is approximately 3%. For a relatively homogeneous bacterial suspension it is about 10% but, as Brown and Broom (1932) have pointed out, the variation which sometimes occurs in the migration rates of individuals in certain bacterial strains derived from cultures of single colonies makes it impossible to obtain a result of any value. Broom and Brown (1932) also when studying the cataphoresis of strains of *C. diphtheriae* noted that certain strains which had shown a constant velocity over a short time interval might migrate with a considerably altered velocity when examined some months later.

TABLE VI

| Date | Velocity in $\mu$/sec volt cm |
|---|---|
| July 11, 1935 ................ | 5·580 ± 0·030 |
| July 12, 1935 ................ | 5·515 ± 0·029 |
| July 15, 1935 ................ | 5·591 ± 0·031 |
| July 16, 1935 ................ | 5·564 ± 0·031 |
| July 17, 1935 ................ | 5·580 ± 0·030 |

When dealing with the cataphoresis of red blood corpuscles, the results have been expressed by Brown (1933, *a*, *b*) and Findlay and Brown (1934) as a ratio between the migration rate of the red cell under investigation and that of a normal red cell of the same species. Their reason for so doing was that it was considered advisable to take samples of normal blood at the same time and expose them to the same conditions as the samples from patients, rather than to assume that the varying uncontrolled factors during transport are of no importance. The evidence at present is insufficient to decide this question.

## THE SIGN AND MAGNITUDE OF THE CHARGE

The determination of the sign of charge of particles presents no difficulty whatever. This can be confirmed by noting the direction of migration of colloidal particles whose charge is of known sign, or by observing the behaviour of bacteria which have been acted upon by suitable concentrations of polyvalent cations.

The position as regards the calculation of the absolute magnitude of the charge is, however, less clear.*

Smoluchowski's equation connecting migration rate and charge of a particle is:

$$\zeta = \frac{4\pi\eta V}{DX}$$

in which

$\zeta$ = the charge of the particle.
D = the dielectric constant of the medium.
$\eta$ = the coefficient of viscosity.
V = velocity in $\mu$ per second.
X = potential gradient.

All electrical units are electrostatic.

According to this theory the velocity is independent of the shape or size of the particles.

Debye and Hückel (1924) came to the conclusion that while the above equation is true for cylindrical particles it requires alteration for spherical particles to

$$\zeta = \frac{6\pi\eta V}{DX},$$

in which case the potential is 1·5 times that given by the previous equation, and is dependent upon the size and shape of the particle.

Abramson and Michaelis (1929), Abramson (1930), and Daniel (1932) performed a number of experiments to test this point and concluded that the size and shape of the particle do not affect the velocity. This conclusion is also supported by the work of Dummett and Bowden (1933). Henry (1931) has re-examined this point mathematically and considers that, subject to certain restrictions, Smoluchowski's equation is justified on theoretical grounds; in a further communication Sumner and Henry (1931) bring forward experimental evidence confirming this contention. Müller (1933) suggests certain refinements to Henry's work but concludes that, although the range within which it is valid cannot be rigidly defined at present, Smoluchowski's formula is probably applicable to any shape or surface within a range when the thickness of the double layer is small compared with the radius of curvature of the particle.

---

* Since this paper was submitted for publication, the question of the relation between particle size and cataphoretic mobility has been critically examined by Kemp (1935). He points out that Henry's equation shows that below a certain particle size Debye and Hückel's equation should hold good, and Smoluchowski's above another value. Between these extremes the velocity should vary with the size. Kemp's experiments show that the intermediate range is of the order of 0·1 to 1·0 $\mu$ particle size.

The findings of the above observers appear, therefore, to favour the acceptance of the Smoluschowski equation rather than the modification suggested by Debye and Hückel.

In the evaluation of the electrokinetic potential from this equation it is necessary to consider both the viscosity $\eta$ and the dielectric constant D of the medium. Kruyt (1930) points out that although it is usual to substitute the values determined for pure water, this could be justified only if $\eta$ and D remain constant when the electrolyte concentration is varied. He states that when the electrolyte concentration is small the effect on $\eta$ may probably be ignored, but that this is not always so with D, the variations of which have not been fully determined.

The above restrictions to the application of this equation need be considered only when one is dealing with the charge of a particle in electrolyte solutions of varying concentrations, since changes in velocity will not necessarily indicate proportional alterations in charge.

Where the same solution is used throughout a series of experiments $\eta$ and D will remain constant though undetermined. Differences in the migration rates of different particles will then be proportional to their relative charges.

We wish to thank Mr. R. J. Reed for the care and patience he expended in making numerous cataphoresis cells. Also Dr. Margaret Zuelzer and Mr. Gustav Fischer for their permission to reproduce Table I from 'Zbl. Bakt.,' vol. **126**, p. 360 (1933).

## SUMMARY

Various discrepancies in the results of different observers appear to us to be due to disregard of certain essential details of technique.

We are of opinion that meticulous attention should be paid to the efficiency of the fine adjustment of the microscope. The slightest irregularity of this mechanism will completely invalidate the results.

A microcataphoresis cell is described which appears to satisfy all requirements when used under the conditions specified.

We have confirmed the value of Komagata's correction of the calculation of the stationary layer for cells in which the width/depth ratio is less than 20.

## REFERENCES

Abramson, H. A. (1930). 'J. Gen. Physiol.,' vol. **13**, p. 657.

—— (1934). "Electrokinetic Phenomena," Chem. Catalog Co., New York.

Abramson, H. A., and Michaelis, L. (1929). 'J. Gen. Physiol.,' vol. **12**, p. 587.

Brown, H. C. (1933, *a*). 'Trans. Roy. Soc. Trop. Med. and Hyg.,' vol. **26**, p. 515.

—— (1933, *b*). 'Brit. J. Exp. Path.,' vol. **14**, p. 413.

Brown, H. C., and Broom, J. C. (1929). 'Brit. J. Exp. Path.,' vol. **10**, p. 219.

—— (1932). 'Brit. J. Exp. Path.,' vol. **13**, p. 334.

Broom, J. C., and Brown, H. C. (1932). *Ibid.*, vol. **13**, p. 337.

Coehn, A. (1898). 'Wied. Ann.,' vol. **64**, p. 217.

Daniel, J. (1932). 'J. Gen. Physiol.,' vol. **16**, p. 457.

Debye, P., and Hückel, E. (1924). 'Phys. Z.,' vol. **25**, p. 49.

Dummett, A., and Bowden, P. (1933). 'Proc. Roy. Soc.,' A, vol. **142**, p. 382.

Ellis, R. (1911). 'Z. phys. Chem.,' vol. **78**, p. 321.

Findlay, G. M., and Brown, H. C. (1934). 'Brit. J. Exp. Path.,' vol. **15**, p. 148.

Henry, D. C. (1931). 'Proc. Roy. Soc.,' B, vol. **133**, p. 106.

Kemp, I. (1935). 'Trans. Faraday Soc.,' vol. **31**, p. 1347.

Komagata, S. (1933). "Res. electrotech. Lab. Minist. Commun., Japan."

Kross, W., and Zuelzer, M. (1932). 'Zbl. Bakt.,' vol. **126**, p. 360.

Kruyt, H. R. (1930). "Colloids," p. 30, London (Chapman & Hall, Ltd.).

Müller, H. (1933). 'Symposia of Quantitative Biology,' vol. **1**, p. 9.

Northrop, J. H. (1922). 'J. Gen. Physiol.,' vol. **4**, p. 29.

Powis, F. (1914–15). 'Z. phys. Chem.,' vol. **89**, p. 91.

Smoluchowski, M. v. (1921). "Graetz's Handbuch der Elektrizität und des Magnetismus," vol. **2**, p. 366.

Sumner, C. G., and Henry, D. C. (1931). 'Proc. Roy. Soc.,' B, vol. **133**, p. 120.

612 . 392 . 61

# Experimental Sodium Chloride Deficiency in Man

By R. A. McCance, Biochemical Laboratory, King's College Hospital

(*Communicated by E. Mellanby, F.R.S.—Received November* 4, 1935)

## Introduction

Dehydration plays an important part in the pathology of a number of diseases. The loss of water may take place through the kidney (as it does in diabetic coma), the gut (as in pyloric stenosis, paralytic ileus, acute diarrhoea, and vomiting or cholera), the lungs and skin (as in hot dry atmospheres) or occasionally through other channels. This loss of water has been known for a very long time to be associated with loss of salt, but a study of the literature reveals considerable confusion of thought as to the relationship of one to the other. This is particularly well illustrated by the discussions of some of the experimental work on Addison's disease. Much experimental work has been done on the lower animals to elucidate the pathology of the intestinal obstructions; diabetic coma has been studied to some extent, and in the last 5 years Addison's disease has been produced and controlled experimentally in numerous rats, cats, and dogs. In every case, however, the salt or water deficiency is but a part of the picture. From the acute and dangerous nature of the human diseases and the complications they introduce it is particularly difficult to study the effects of severe water or salt deficiency in patients. Some work has been carried out on diabetic coma, which, however, presents water and salt loss in its most complicated form. No papers of any value have been found on severe uncomplicated salt deficiency. It was therefore decided to make a direct experimental attack on the question and normal human adults were selected as the most suitable subjects. The deficiency was produced by a salt free diet combined with sweating.

## Nature and Arrangement of the Experiments

(*a*) *The Subjects*—One of the women students of this Hospital volunteered to be the subject for the first experiment, which was of a semi-quantitative nature and was intended to try out methods rather than get results. Mild deficiency only was produced because E. ceased to lose significant amounts of NaCl in her sweat after 4 or 5 days. Nevertheless, some interesting observations were made which will be discussed in their appropriate place, and the fact that this subject reacted to the experimental regime so differently from the others is a matter which may be of some importance and will be further investigated. The second and third experiments were done on two males, R.A.M. age 36, and R.B.N. age 24. In both experiments every reasonable precaution was taken to make the whole investigation as quantitative as possible. Both subjects were in good health when the experiments began and the observations were not upset by any " colds " or other minor pathological interferences. R.A.M. is normally an active man, fond of exercise, and gets enough of it throughout the year to keep himself physically fit. R.B.N. is a South African, who came to this country with a Rhodes Scholarship and is now a medical student. He takes enough regular exercise to keep in fair training.

(*b*) *Sweating*—This was carried out in a radiant heat bath. A mattress with two pillows at one end was placed on the floor and covered by a strip of mackintosh sheeting 8 feet long. The bath, which consisted of a metal and wood tunnel closed at one end and fitted internally with three rows of electric hot lamps, was placed on the mattress and the open end covered by hanging a small piece of sheeting over it. When the temperature was fully raised the subject entered feet first through the open end and lay there. While in the air bath he drank a measured amount of water, made notes and regulated his own temperature by raising or lowering the flap of sheeting. After 15-25 minutes sweat poured off and an assistant was always within call to help to bail this off the sheeting. 800 cc were often collected in this way. After 2 hours the heating was switched off and 10 minutes later the subject crawled out and was washed down quantitatively with a jet of warm distilled water into an enamel washing tub. The sheetings and parts of the air bath in contact with them were subsequently washed down with distilled water into the tub. The contents of this were evaporated down at a neutral or slightly acid reaction, combined with the liquid sweat which had been bailed off the sheeting during the time the subject had been in the bath, and made up to a convenient volume, generally 2 litres. The condition of the subject in the bath is best described by one of the records made at the time:—

*Subject R.A.M.*, 10.1.35—

2.16 p.m.—Naked weight 9 st 12 lb 0 oz.
2.18—Into bath. Temperature 98·0°. Begin drinking 800 cc water.
2.25—Moist all over, lying on face.

2.33—Temperature 99·2° sweating well. Drinking.
2.45—Temperature 100°, sweating very well. Always seem to sweat well when temperature rising. First bail out.
2.55—Temperature 100·8°. Drinking.
3.10—Temperature 101°. Sweating very well.
Half-time—Temperature 101°. Comfortable.
3.30—Temperature 101·6°. Too hot, uncomfortable, pulse banging. Open flap.
3.35—Temperature 101·4°. Water nearly finished.
3.45—Temperature 101°. Water all done. Not sweating so well, still uncomfortable. Sickish.
3.50—Better, sweating well. Temperature 100·8°.
4.10—Temperature 101°. O.K. but cramps.
4.20—Heat off.
4.30—Out, feel rotten few minutes, washed down. Then O.K. Weight before dressing 9 st 9 lb 0 oz. Total loss of weight = 3 lb + 800 gm = 2150 gm.

(*c*) *Diet and mode of life*—R.A.M. lived at his own home and R.B.N. stayed there also during his experiment. Both subjects shaved every day, taking great care to remove all the lather, but no other " domestic " washing or soap of any kind was indulged in during the experiment except for the hands. Their food, which was carefully prepared and accurately weighed, consisted of salt-free " casein " bread, synthetic NaCl free milk, salt-free butter, fruit, thrice boiled vegetables, jam, home-made salt-free shortbread and toffee. The milk and bread were devised for these experiments (Widdowson and McCance, 1935) to enable a reasonably high protein intake to be combined with a salt-free diet, and both proved most satisfactory. R.A.M. experimented with washed mince made up with curry as a variety, but this proved so nauseating that he fell back entirely on to the casein bread and special milk for his supply of protein. Water was taken as desired by all the subjects.

At the end of her deprivation period E. took 28 gm NaCl in a few hours, drank copiously for the next 12 hours, and was back to normal activity and weight the following morning. This meant an increase of 7¼ lb. R.A.M. brought himself slowly back to health by eating regulated quantities of anchovies, bacon, liver, measured amounts of cow's milk, and small weighed amounts of NaCl. Every third rasher of bacon was taken for analysis in the raw state, and to prevent loss, all bacon was eaten from the frying pan used for its preparation, and the pan washed out with hot water which was drunk. Samples of the anchovies were also analysed. All other food was maintained practically salt free, but ordinary salt-free bread was taken instead of the casein-enriched bread, since plenty of protein was now being eaten. R.B.N. used bacon, roast beef, and small amounts of NaCl to remove his salt deficiency. He observed the same precautions as R.A.M. and changed also from " casein " to ordinary bread.

Except for these salted foods used during the recovery period and special foods such as the washed mince, which were all analysed for nitrogen, potassium, sodium, and chloride, the composition of the foods eaten was calculated from the tables of McCance and Shipp (1933) and unpublished data of McCance, Widdowson, and Shackleton.

(*d*) *Collection and treatment of excreta*—Urines were collected and analysed in 24-hour periods, or shorter if kidney function tests were being made. Faeces were collected and analysed in 3- or 4-day periods. To obviate loss of nitrogen, the portion of moist faeces which had been weighed out for analysis was well mixed with glacial acetic acid before being placed in the oven at 50° C to dry. The portion to be used for chloride determinations was dried without any additions. The sweat and bath washings were collected for analysis as already described. The insensible perspiration was collected in the following way:—A set of underclothes was washed in distilled water till no reaction for chlorides was obtained from the washings. They were then dried and put on when the experiment began. All R.A.M.'s clothes were washed in this way, but only underclothing in the case of R.B.N. These underclothes were worn night and day throughout the period of deprivation and were only removed for the periods of sweating in the air bath. They were then washed out as before, dried in a few hours and worn continuously till the recovery period was over, when they were washed again. The washings were evaporated and analysed. At the conclusion of the recovery period the subjects were washed down with distilled water and the washings combined with those from the underclothes. Owing to the regular washings after the sweat baths this additional wash down was omitted at the close of the deprivation period.

## METHODS

1. *General*—

Blood pressure. Sphygmomanometer of usual type with mercury column.

Eye tension. McLean's tonometer.

2. *Chemical methods for urine*—

Total nitrogen. Kjeldahl (McCance and Shipp, 1933).

Urea. A modification of Beattie's (1928) xanthydrol procedure.

Creatinine and creatine. Folin (Hawk, 1923).

Chlorides. Volhard's principle; the urine was heated at 100° C with $HNO_3$ and excess $AgNO_3$ (McCance and Shipp, 1933).

Metallic radicles. After incineration. McCance and Shipp (1933). When a large excess of potassium over sodium was present the former was removed with perchloric acid before determining the latter (McCance, Widdowson, and Shackleton).

3. *Blood*—

Cell volume. Haematocrit, coagulation prevented by minimal amounts of ammonium oxalate.

Haemoglobin. Haldane's CO technique.

Urea. A modification of Beattie's (1928) method.

Serum proteins. Kjeldahl (McCance and Watchorn, 1931) or micro-Kjeldahl (Pregl).

Alkali reserve. Van Slyke gasometric (Harrison, 1930).

Plasma chlorides and whole blood chlorides. Volhard's principle, Van Slyke (1923–24) and McCance and Shipp (1933). Plasma for chlorides and alkali reserve was taken under paraffin without constriction.

Sodium in serum. A less micro form of the method described by McCance and Shipp (1931).

Potassium in serum. Kramer and Tisdall (1921), Hubbard (1933).

Osmotic pressure of the serum by depression of freezing point using a standard Beckmann thermometer.

Metallic radicles in whole blood. Incineration (McCance and Shipp, 1933).

4. *Sweat, washings, etc.*—As urines.

## SYMPTOMS AND SIGNS

As the deficiency developed all three subjects lost weight (*see* later). Their cheeks fell in and they began to look ill. People who had no idea that they were being used as experimental animals commented on their appearance. Their sense of flavour and taste was affected. E. interpreted this aberration or lack of sensation as thirst. She complained of it constantly and drank freely but without obtaining any relief. R.A.M.

recognized the feeling as distinct from thirst. His mouth was not unduly dry but food was tasteless, even highly flavoured food, and this was the more noticeable because such foods were eagerly sought to make the meals more appetizing. Chewing fried onions, for example, evoked only a sensation of greasy sweetness which was extremely nauseating. The distaste, however, was not confined to meals and was a feature of every waking hour " Even cigarettes don't taste."* R.B.N. was not so much troubled by this symptom but felt it from time to time. He noted once that he was " Thirsty all morning—drank a lot but water seems to make little difference " and on another day reported that he had a " funny feeling in the mouth."*

On the whole all slept well but R.A.M. and R.B.N. were apt to be roused by attacks of nocturnal diuresis, and both were troubled by nightmares. Both were probably in a fairly exhausted state by the end of the deprivation period, and R.A.M. went to sleep in the laboratory about an hour and a half after his first meal containing salt. R.B.N. and R.A.M. both found it very difficult to get through their prescribed rations. R.A.M. was " never hungry "* but preferred the bread to anything else, while R.B.N. found it almost uneatable towards the end. " It's the bread that gets me down."* Nausea accompanied almost every meal but neither subject had indigestion after meals or any constipation. R.A.M. suffered considerable abdominal discomfort during and after the hot air baths on several days, but food tended to relieve it and this was certainly not the cause of the nausea.

Both the male subjects suffered considerably from cramps, but E. escaped, probably because her deficiency was relatively slight. These muscular cramps were not of the very severe localized type which are said to affect stokers and miners (Moss, 1923–24), but were widespread, frequent, not very painful, and generally controllable. Any muscle in the body was liable to go into spasmodic contraction, especially if some little effort was demanded of it (Moss, 1923–24). " Coughed a little over something, cramps round chest, mild."* R.B.N. experienced occasional cramps in the muscles lining the floor of the mouth but the extremities were most affected, and it was possible sometimes by suitable movement of the toes to alternate the spasms for hours on end between the extensor and flexor muscles. Perhaps the most characteristic of all were the manual cramps. R.A.M. experienced " constant mild cramps of the fingers and thumb when using forceps at the balance,"* and R.B.N. found that he " cramped quickly when working, telephoning, or using pipettes."*

---

* Diary.

Physical symptoms predominated in the case of R.A.M. but all experienced them in some degree. A mild breathlessness at first and sense of fatigue gave place later to general exhaustion and distress on the least exertion. The effort of dressing, breakfasting, and getting into and out of a car was so great that R.B.N. " lay exhausted on a couch in the common room on arriving."* R.A.M. found that going up two flights of stairs to the laboratory was a serious undertaking, causing a sense of breathlessness and a most unpleasant feeling of constriction across the sternum which compelled him to stop and rest. Throughout the experiment he used to go for a measured walk of about a mile after breakfast. Towards the end of the deficient period the breathlessness and sense of constriction forced him to sit down and rest two or three times at a hill, for which he would ordinarily not have slackened pace. Little acts of the daily routine produced a localized sense of fatigue; his " arm got tired shaving "* and finally his " jaw got tired eating toast."*

Mentally R.A.M. felt normal but R.B.N. felt " slow in the head "* and showed it in his behaviour. He would remain in a chair for periods of 30–60 minutes " content to sit and do nothing."* He never slept on these occasions and was always willing to talk, but spoke slowly and rarely initiated conversation. For several days he experienced at frequent intervals sensations of " *déjà vu.*" He became apathetic and his mental processes appeared to be dulled. R.B.N. longed for salt and often went to sleep thinking about it. R.A.M. felt no specific craving for salt and had difficulty in convincing himself that taking salt would at once make him feel all right again.

In both subjects the resting pulse rate remained normal, but the volume became very small. The pulse during exercise was not investigated. The blood pressure was taken on numerous occasions in the case of R.B.N. and rather less frequently in the case of R.A.M. Both subjects had normal blood pressures and maintained them within narrow limits throughout the experiment. No fall of systolic or diastolic pressure accompanied the salt deficiency and actually the lowest figure in each subject was obtained under normal conditions.

The eye tension of all three subjects was investigated when they were normal and at the height of the deficiency. The results which are shown in Table I indicate no consistent change. Temperatures were taken in the mouth before and during the hot air baths. They were always within normal limits before entering the bath (Marriott, 1923).

Recovery was quite dramatic. Half an hour after eating 15 gm of

---

* Diary.

NaCl with bread, butter, and an egg E.'s sense of flavour and taste had returned, although no fluid had been taken. This she spoke of as a quenching of her thirst. Genuine and almost unbearable thirst supervened later and was only satisfied by copious draughts of water. R.A.M. found his sense of flavour returned before he had finished his first salt meal. About an hour later he felt hot all over, he experienced a deep seated pricking and tingling in his extremities and was conscious of bounding pulsation. In a few hours he was much more comfortable in mounting the stairs, and by evening was " no longer aware of his legs as he moved about the room."* His carriage and movements were observed to be more normal. He felt more " himself," and on the next day although not yet normal, he experienced an " exhilarating sense of well-being "* from morning till night.

TABLE I

| | Normal mm Hg | During deficiency mm Hg |
|---|---|---|
| E. .................... | 35 | 25 |
| R.A.M. .............. | 32 | 30 |
| R.B.N. ................ | 25 | 32 |

R.B.N. ate his first meal containing salt in the evening and made no comment on the return of flavour and taste, but this is not surprising because this symptom had never worried R.B.N. very much. He did, however, feel as though " his heart was beating all over him " and his vaso-motor reactions were no doubt very similar to those of R.A.M. During the night he had some colic and diarrhoea, and was still breathless and very easily tired the next day. After 48 hours he " jumped off the 'bus while it was going, and ran up the stairs "*—simple pleasures, but keenly enough appreciated to make him record that he had " had a grand day."*

### *The Blood*

All three subjects have shown similar changes, the main differences being those of degree. The appearance and behaviour of the venous blood altered as the experiment proceeded. It became dark and viscous and would not flow satisfactorily even through a large bore needle, so that some difficulty was experienced in getting the necessary samples. The red cell counts, the haemoglobin and the plasma proteins rose, and with them presumably the colloidal osmotic pressure of the plasma. The concentrations of sodium and chloride in the plasma fell, and also the total osmotic pressure as indicated by a smaller depression of the freezing point. Such a syndrome has become a familiar one since experimental suprarenalectomies have been undertaken. Further, the concentration of urea in the blood rose, but not enough to maintain the total O.P. of the plasma. The alkali reserve did not change, nor did the serum potassium. There was a decrease in the corpuscular chlorides. With recovery, all the crystalloids were restored to their initial values, but the cell counts, haemoglobin and plasma proteins became subnormal for a time. The actual changes observed in R.B.N.'s experiment are shown in Table II. The depression of the freezing point was not determined in his serum. In R.A.M. it rose from −0·596° C before the experiment began to −0·547° at the close of the deprivation period, and fell again to −0·615° during recovery. In E. the changes were in the same direction but smaller, and since the chemical changes of R.B.N. resembled closely those of E. and R.A.M. the determination of the F.P. was omitted. In one small respect the serum changes of the three subjects have not agreed. Considered in terms of equivalent weights R.B.N.'s chlorides fell more than his sodium. With R.A.M. and E. the reverse occurred. The greatest care was taken over the determinations and no technical reason for these differences could be found.

---

* Diary.

### *The Nitrogen and Potassium Balances*

Balances of nitrogen, potassium, sodium, and chloride were determined on R.A.M. and R.B.N. but not on E. In both, sodium chloride deficiency produced a negative nitrogen balance which was partially masked by the retention of nitrogenous waste products as exemplified by the rise of blood urea. R.B.N.'s nitrogen balance as determined by experiment is given in Table III and it will be noticed that there was a negative balance of some 36 gm which was converted to a small positive balance during recovery. It must be emphasized that both subjects were on a carefully weighed diet and that ample food was eaten to supply their calorific requirements, so that undernutrition may be excluded as a cause of the loss of body nitrogen. The balance corrected for urea retention is shown in Table IV and indicates that 45 gm of nitrogen were lost during the deprivation and between 12 and 13 gm regained during the 7 days' recovery. Table V shows the apparent (uncorrected) nitrogen balance of R.A.M. made up in two- and three-day periods and this illustrates rather well the fact that the balance steadily became more negative as NaCl was withdrawn from the body. The corrected balance would demon-

TABLE II—R.B.N.

| Period | Cell count mil/cmm | Haemoglobin % of normal | Cell vol % | Serum proteins % | Urea mg/100 cc | Chlorides mg/100 cc |  | Alk. res. vols % | Serum mg/100 cc |  |
|---|---|---|---|---|---|---|---|---|---|---|
|  |  |  |  |  |  | Plasma | Corpuscles |  | Na | K |
| Before experiment .. | 5·2 | 101 | 44 | 6·4 | 31 | 361 | 204 | 69 | 340 | 19·4 |
| Day of deprivation— |  |  |  |  |  |  |  |  |  |  |
| 9th .................. | 6·4 | 125 | 56 | 8·0 | 71 | 291 | 168 | 73 | 308 | 19·3 |
| 10th ............... | 5·9 | 115 | 56 | 7·7 | 84 | 275 | 160 | 67 | 302 | 16·2 |
| 11th and last ...... | 6·1 | 117 | 52 | 7·2 | 69 | — | — | — | — | — |
| After recovery ...... | 4·5 | 93 | 40 | 6·1 | 25 | 361 | 218 | 66 | 342 | 15·0 |

strate this even more clearly. R.A.M. lost 33·6 gm of body nitrogen (corrected) during the deprivation period as against R.B.N.'s 45 gm and showed a small negative balance instead of a positive one over the whole recovery period. This last is explained by the fact that R.A.M.'s recovery period lasted only 5 days while R.B.N.'s lasted 7. In all other respects the balances closely resembled each other, and may be considered in good agreement as regards essentials.

TABLE III—NITROGEN BALANCE. R.B.N.

| Day | Intake | Output | | | | |
|---|---|---|---|---|---|---|
| | | Urine | Faeces | Sweat | Insensible perspiration | Blood (experimental) |
| | gm | gm | gm | gm | gm | gm |
| | | *Deprivation Period* | | | | |
| 1 | 16·3 | 14·8 | 1·05 | 0·88 | | |
| 2 | 12·9 | 14·4 | 1·05 | 0·52 | | |
| 3 | 13·7 | 13·1 | 1·05 | 0·59 | | |
| 4 | 13·7 | 13·7 | 1·05 | 0·48 | | |
| 5 | 12·6 | 13·6 | 1·2 | 0·68 | | |
| 6 | 13·1 | 15·8 | 1·2 | 0·62 | | |
| 7 | 13·4 | 10·6 | 1·2 | 0·81 | | |
| 8 | 13·7 | 16·3 | 1·1 | 0·67 | | |
| 9 | 15·3* | 17·6 | 1·1 | 0·85 | | |
| 10 | 15·2* | 21·2 | 1·1 | | | |
| 11 | 17·0* | 23·0 | 1·2 | | | |
| | | 174·1 | 12·3 | 6·1 | 0·55 | 8·5 |
| Total...... | 156·9 | 193·1 | | | | |
| | | *Recovery Period* | | | | |
| 1 | 14·5 | 19·0 | 1·20 | | | |
| 2 | 15·1 | 16·6 | 1·20 | | | |
| 3 | 15·0 | 14·0 | 1·22 | | | |
| 4 | 17·6* | 14·2 | 1·44 | | | |
| 5 | 15·6 | 10·4 | 1·44 | | | |
| 6 | 15·1 | 10·0 | 1·44 | | | |
| 7 | 15·6 | 10·6 | 1·44 | | | |
| | | 95·4 | 9·4 | | 0·6 | 1·2 |
| Total...... | 108·5 | 105·4 | | | | |

* 1·8 gm of the intake on these days consisted of nitrogen taken for purposes of studying kidney function.

The extra nitrogen appeared to be excreted as urea. A full nitrogen partition of the urine was never investigated, but the ammonia was determined on 7 random days during R.A.M.'s experiment and found to lie between 0·5 and 0·8 gm per day, which is a normal range. The creatinine was determined every day. The results are given in Table VI as creatinine N. and show that the daily excretion maintained its normal constancy. Hartwell and Hoguet (1912) had recorded that dogs with

TABLE IV—CORRECTED NITROGEN BALANCE. R.B.N.

| | Intake total gm | Output observed gm | Catabolized but retained gm | Catabolized and excreted gm | Balance gm |
|---|---|---|---|---|---|
| Deprivation period.... | 156·9 | 193·1 | 8·8* | 201·9 | −45·0 |
| | | | Excreted but not catabolized | Excreted less non-catabolized nitrogen | |
| Recovery period .... | 108·5 | 105·4 | 9·7* | 95·7 | +12·8 |

* These figures are arrived at by assuming (*a*) that 60% of the total weight of the body is water; (*b*) that the concentration of urea found in the blood holds throughout all the water in the body.

TABLE V—APPARENT NITROGEN BALANCE. R.A.M.

| Days | Daily intake (average) gm | Daily output* (average) gm | Balance gm |
|---|---|---|---|
| | *Deprivation Period* | | |
| 1–3 inclusive .............. | 13·5 | 13·4 | +0·1 |
| 4–6 inclusive .............. | 12·9 | 14·4 | −1·5 |
| 7–9 inclusive .............. | 14·1 | 16·5 | −2·4 |
| 10–11 inclusive† ............ | 13·2 | 16·5 | −3·3 |
| | *Recovery Period* | | |
| 1–3 inclusive .............. | 18·5 | 22·4 | −3·9 |
| 4–5 inclusive .............. | 17·9 | 16·3 | +1·6 |

* The output includes all sources except blood taken for analytical purposes.

† The last day of the deprivation period was not a full 24 hours. For the construction of this table the actual output for 19 hours has been multiplied by 24/19. The intake for the missing meal has been assumed to be the same as the intake on the previous day.

Applying a correction for the retained urea would increase the negative balance of the latter part of the deprivation period and decrease the negative balance of the early part of the recovery period.

intestinal obstruction excreted large amounts of creatine, and this substance therefore was looked for on several days, but was found to be absent, or present in traces only. Much more urea, however, was excreted as the deficiency developed, and further, the urea nitrogen expressed as percentage of the total nitrogen tended to rise as the deficiency developed and fall again with recovery.

TABLE VI—DAILY EXCRETION OF CREATININE N. R.A.M.

| *Deprivation Period* | | | | *Recovery Period* | |
|---|---|---|---|---|---|
| Day | Creatinine N excreted gm | Day | Creatinine N excreted gm | Day | Creatinine N excreted gm |
| 1 | 0·60 | 6 | 0·55 | 1 | 0·62 |
| 2 | 0·56 | 7 | 0·62 | 2 | 0·57 |
| 3 | 0·58 | 8 | 0·57 | 3 | 0·62 |
| 4 | 0·55 | 9 | 0·61 | 4 | 0·62 |
| 5 | 0·58 | 10 | 0·54 | 5 | 0·60 |
| | | 11* | 0·49 | | |

* Excreted during 19 hours.

A large negative nitrogen balance would almost certainly be accompanied by a negative potassium balance. A loss of 45 gm of nitrogen from muscle might be expected to set free about 4500 mg of potassium, an amount rather difficult to detect under these experimental conditions, where the intake was of the order of 33,000 mg. R.B.N.'s potassium balance is shown in abridged form in Table VII and while no stress can be laid upon the result it shows a negative balance of 3600 mg during the experimental period and a positive balance of 580 mg during recovery. These figures are of the right order to tally with the nitrogen balances. R.A.M.'s potassium balance did not show such close agreement with his nitrogen balance. There was a negative balance of 560 mg during the deprivation period and a positive balance of 1660 mg during recovery, results qualitatively agreeing with those of R.B.N. but possibly of no significance. The question of the relation of the potassium to the nitrogen balance must be left for the time being *sub judice*.

### *The Sodium and Chloride Balances*

Table VIII shows the sodium balance obtained on R.A.M. During the deprivation period the intake was about 40 mg a day. The output from all measured sources is shown in detail and the relative importance

TABLE VII—POTASSIUM BALANCE. R.B.N.

| | Total intake mg | Output mg | | | | | | Balance |
|---|---|---|---|---|---|---|---|---|
| | | Urine | Faeces | Sweat | Insensible perspiration | Blood for experimental purposes | Total (excluding blood) | |
| Deprivation period (11 days) | 33,244 | 24,645 | 7250 | 4550 | 417 | 370 | 36,862 | −3618 |
| Recovery period (7 days).. | 24,342 | 18,060 | 5010 | — | 692 | 72 | 23,762 | +580 |

of each may be seen by inspection. The net loss was 17,540 mg. It is unfortunately very difficult to assess the total amount of sodium in an adult's body, but there is no doubt that 17,540 mg is a large fraction of the whole (*vide infra*). The net gain during the recovery period exceeded the loss during the deprivation period by 3525 mg. R.A.M., however, was 1·03 kg heavier at the close of the experiment than he was when it began. It is most unlikely that this was a gain in weight of muscle or of any other organ owing to the simultaneous loss of nitrogen. It may have been fat but there is no evidence for this and was much more probably extra-cellular water (Adolph, 1921) and this is supported by the dilution

TABLE VIII—SODIUM BALANCE. R.A.M.

*Deprivation Period*

| Day No. | Intake mg | Output, mg | | | | |
|---|---|---|---|---|---|---|
| | | Urine | Faeces | Sweat | Insensible perspiration | Blood (for tests, etc.) |
| 1 | 58 | 3160 | 125 | | | |
| 2 | 40 | 1300 | 125 | 2680 | | |
| 3 | 40 | 378 | 125 | 3000 | | |
| 4 | 51 | 133 | 125 | | | |
| 5 | 51 | 66 | 50 | 2500 | | |
| 6 | 34 | 31 | 50 | | | |
| 7 | 35 | 26 | 50 | | | |
| 8 | 41 | 25 | 50 | 1900 | | |
| 9 | 38 | 11 | 76 | | | |
| 10 | 36 | 17 | 76 | 880 | | |
| 11 | 34 | 10 | 76 | | | |
| Totals .... | 458 | 5157 | 936 | 10,960 | 690 | 255 |
| | | | | 17,998 | | |

Net loss: 17,540.

*Recovery Period*

| Day No. | Intake mg | Urine | Faeces | Sweat | Insensible perspiration | Blood (for tests, etc.) |
|---|---|---|---|---|---|---|
| 1 | 5911 | 22 | 43 | | | |
| 2 | 5599 | 22 | 43 | | | |
| 3 | 5408 | 370 | 43 | | | |
| 4 | 5899 | 1450 | 43 | | | |
| 5 | 5218 | 4520 | 44 | | | |
| | 28,035 | 6384 | 216 | — | 370 | — |
| | | | | 6970 | | |

Net gain: 21,065.

of the blood towards the close of the recovery period. A gain of 1·03 kg of extra-cellular water would require a retention of about 3400 mg of sodium, so that the corrected balance sheet agrees to a few mg. Thus:—

| | mg | | mg |
|---|---|---|---|
| Net loss .............. | 17,540 | Net gain during recovery | 21,065 |
| | | Deduct for gain in weight of 1·03 kg .......... | 3,400 |
| | 17,540 | | 17,665 |

R.B.N.'s balance was also a good one. A net loss of 22,516 mg took place during the deprivation period which was greater than R.A.M.'s but considered in relation to his greater body weight, the two results are nearly the same. A gain of 21,514 mg accompanied recovery. There was, therefore, a negative balance of 1002 mg on the whole experiment. R.B.N. lost 1·06 kg during this time, but since there was also a loss of 30 gm of nitrogen, some of this loss of weight may have been loss of muscular tissue. No correction, therefore, for the change in weight has been applied.

Table IX shows R.A.M.'s chloride balance expressed in terms of Cl. A loss of 20,577 mg of chloride during deprivation was followed by a gain during recovery of 30,296, and even correcting the latter for the increase in weight, the gain during recovery comes to 26,696 mg, considerably more than the previous loss. Considering the care with which the experiment was conducted and the proximity of the sodium balance, this discrepancy cannot be attributed to experimental error. R.B.N.'s chloride balance, moreover, was essentially the same in all important respects and showed a similar discrepancy. The facts may be presented in another way as has been done in Table X. There it will be seen that in both subjects the *losses* of the two elements proceeded *pari passu* with their relative concentrations in serum, *i.e.*, more sodium than chloride was lost. During recovery neutral sodium chloride was taken by mouth and it was this salt, *i.e.*, sodium and chloride in equivalent amounts, which was taken up and retained by the tissues. Thus it follows that 160 milli equivalents of sodium in R.A.M.'s body, which had at the beginning of the experiment been combined with ions other than chloride, were combined with this ion as the neutral salt when the experiment terminated. The excess of chloride was no doubt excreted as the ammonium salt as time went on. It has been pointed out by a number of people that the basic ions are of much greater

### TABLE IX—CHLORINE BALANCE. R.A.M.

*Deprivation Period*

| Day No. | Intake as Cl mg | Output as Cl, mg | | | | |
|---|---|---|---|---|---|---|
| | | Urine | Faeces | Sweat | Insensible perspiration | Blood |
| 1 | 90 | 3900 | 84 | | | |
| 2 | 224 | 1200 | 84 | 3460 | | |
| 3 | 165 | 550 | 84 | 3800 | | |
| 4 | 359 | 79 | 84 | | | |
| 5 | 284 | 42 | 55 | 3430 | | |
| 6 | 141 | 24 | 55 | | | |
| 7 | 173 | 30 | 55 | | | |
| 8 | 212 | 26 | 55 | 2770 | | |
| 9 | 153 | 12 | 42 | | | |
| 10 | 244 | 24 | 42 | 1230 | | |
| 11 | 118 | 11 | 42 | | | |
| | 2163 | 5898 | 682 | 14,690 | 1080 | 390 |

22,740

Net loss: 20,577

*Recovery Period*

| Day No. | Intake as Cl mg | Urine | Faeces | Sweat | Insensible perspiration | Blood |
|---|---|---|---|---|---|---|
| 1 | 9185 | 22 | 79 | | | |
| 2 | 8474 | 110 | 79 | | | |
| 3 | 8224 | 760 | 79 | | | |
| 4 | 9137 | 3400 | 79 | | | |
| 5 | 8163 | 7530 | 79 | | | |
| | 43,183 | 11,822 | 395 | — | 670 | — |

12,887

Net gain: 30,296.

### TABLE X

All figures for losses and gains are given in milli equivalents

| | *Deprivation Period* | | | | *Recovery Period* | | | |
|---|---|---|---|---|---|---|---|---|
| | Na lost | Cl lost | Na/Cl ratio | Na/Cl ratio in serum | Na gained | Cl gained | Na/Cl ratio | Na/Cl ratio in NaCl |
| R.A.M..... | 765 | 590 | 1·30 | 1·41 | 770 | 750 | 1·02 | 1·00 |
| R.B.N..... | 980 | 760 | 1·29 | 1·41 | 940 | 980 | 0·96 | 1·00 |

significance to the body than the acidic, and these results provide further evidence that this is so.

### *The Water Balance*

Water was taken as desired by all three subjects, and the urine volumes were from 1500 to 2000 cc per day. No attempt was made to measure the intake and output of water owing to the difficulty in assessing the amount taken with the food and of measuring the amount lost through the lungs, but in short term experiments such as these the fluctuations in body weight may be taken as a rough measure of the variations in body water. The weights were always taken for this purpose under standard conditions—just before lunch, naked, and with an empty bladder—and all three subjects showed, as was to be expected, a considerable loss. Since this loss of water was brought about solely by a loss of salt (or more correctly of sodium ions) the relation of the former to the latter is important, and is shown in fig. 1. The two scales are so constructed that a loss of 1 kg of water is equal to a loss of 3300 mg of sodium, which is the amount of sodium present (approximately) in a litre of serum or other extra-cellular fluid. It will be noted that at first a removal of sodium was

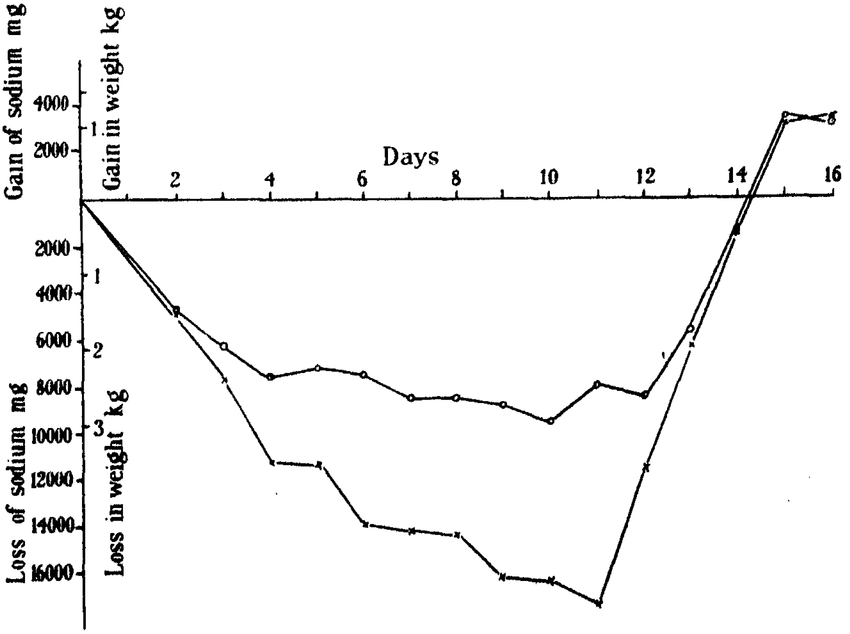


Fig. 1—The weight scale and the sodium scale are so constructed that 1 kg in weight = 3300 mg of sodium. o weight; × sodium.

followed *pari passu* by a loss of an equivalent amount of water. So long as this was so, red cell counts may have risen and other signs of anhydraemia appeared, but *no change in the osmotic pressure* of the body fluids had taken place. Further forced removal of sodium was *no* followed by an equivalent loss of water. The volume of the body fluids was relatively well maintained but *only at the expense of the osmotic pressure*, which fell. Normal fluid regulation (Adolph, 1921) during this stage was noted by both R.A.M. and R.B.N. to be very defective. Copious drinks of water were apt to be followed by no diuresis at the expected time. Hours later, during the night or even the next day, the diuresis would begin. This gave rise to curious weight fluctuations of which the rise in weight between days 10 and 11 is an example. R.B.N.'s weight altered widely from day to day during the worst of his deficiency and the changes bore no relation to those of the sodium. The first effect of restoring sodium was to produce a loss of weight (day 12). R.B.N.'s loss was much greater at this stage and his lowest weight was recorded the day after he began taking salt. Further restoration of salt led to minor retentions of water, till the level was reached at which the salt and water losses had parted company. After this point their restoration proceeded together.

## DISCUSSION

Before opening any discussion it is most important to emphasize once again that water was freely taken by all the subjects of these experiments, and that the urine volumes were large, considering the amount of water lost in the sweat. Loss of water therefore was fully compensated and the experiments show the effects of salt loss without water loss. Such clear cut experimental conditions have never been met with in man and have seldom been achieved in animals, and it is clear from the occasions when they have been, that there are probably important differences in the way in which lower orders react. Rabbits, for example, lose no weight (Michelsen, 1933). The mental confusion that undoubtedly exists over the relationship between salt loss and water loss and their effects must be attributed to the fact that most attempts to study the problem have been made on patients or animals suffering from conditions in which there was a forced loss both of salt and water and in which the latter was not fully replaced. It is hoped to review the whole literature bearing on this question in the Goulstonian lectures in 1936.

From fig. 1 it is clear that in man the removal of salt leads first to a loss of body water with little change in extra- or intra-cellular osmotic pressure. Some rise of red cell count and concentration of the organic constituents of the plasma probably take place. Further withdrawal *forces the body into a compromise between (a) the maintenance of the total osmotic pressure of the extra-cellular fluids, which must lead to progressively severe anhydraemia and a steady rise in the colloidal* O.P. of the plasma; (*b*) the maintenance of the plasma volume at the expense of a fall in its total O.P., which may lead to a fall in the total and colloidal O.P. of the cell fluids. The red blood cells may swell owing to their hypotonic surroundings (Lavietes *et al.*, 1935) and further embarrass the capillary circulation, and, so far as space allows them, other cells of the body may also swell. Thus in experimental suprarenal deficiency (Loeb *et al.*, 1935), when the blood changes are very similar to those met with in the present experiments, the muscles and other organs have been found to contain more water than the controls and the excretion of ingested water to be very much delayed and abnormal (Silvette and Britton, 1933; Silvette, 1934; Swingle *et al.*, 1934). It may have been changes of this sort which caused the large weight fluctuations during the worst period of R.B.N.'s deficiency and it may have been the return of intracellular fluid to the vessels and its excretion which caused both subjects to lose weight on first taking salt. If the total osmotic pressure of the plasma falls and the cells are unable to swell, then an O.P. gradient must be set up between the cells and their surroundings (assuming for the moment no passage of kations takes place across the cell membranes). Neither the anhydraemia nor the loss of weight are in themselves any measure of the degree of a sodium deficiency. The anhydraemia may even improve for a time (and the weight increase) if the organism is prepared to put up with a further fall in its total extracellular, and possible intracellular, osmotic pressure.

These results have practical application. The loss of body weight which can be produced by sweating is limited in amount. Boxers and rowing coxes who may resort to this method should not only realize this, but also that even a small reduction produced in this way may be very detrimental to efficiency.

### *The Symptoms and Signs*

The rapid restoration of the sense of flavour on taking salt points to its loss being one of the effects of hyponatraemia or hypochloraemia. It is probably not connected with the anhydraemia. Clinical observation supports this. Patients with intestinal obstruction complain of dreadful thirst. It is said that small injections of hypertonic saline,* too small to have any effect on the general anhydraemia, may relieve the patient's symptom which may have been not so much thirst as this aberration of sensation. The cramps are probably allied to the so-called stokers' cramps and may also be attributed to the low osmotic pressure of the serum. The cardiovascular symptoms should probably be regarded as the result of the anhydraemia, but very little is known as yet of the effects of anhydraemia without simultaneous sodium deficiency. The reduction in blood volume, however, and the increased viscosity of the blood must decrease the efficiency of the gas exchanges, for (1) the circulation rate may be lowered, a matter which is being investigated; (2) only a fraction of the normal number of capillaries may be in action; (3) there may be some anoxic anoxaemia. The oxygenation of the venous blood is certainly subnormal (Haden and Orr, 1927, *b*). The arterial blood has not yet been investigated in man, but Herrin (1935) has found it to be incompletely oxygenated in dogs under similar conditions. The breathlessness, distress, and fatigue can be explained along these lines. It is interesting to speculate how much of the Addisonian asthenia and fatigue may be due to the sodium deficiency and how much, therefore, of the Addisonian syndrome may be experimentally reproduced in this way in man. The symptoms and many of the signs are undoubtedly very similar but there are also points of difference which are equally significant. Experimentally, for example, the blood pressures do not seem to fall. Symptoms of minor salt deficiency appear to be common enough in the tropics (McEwen, 1935).

---

* The author is indebted to Mr. J. B. Hunter for this observation.

### *The Negative Nitrogen Balance*

This is probably the result of the cardiovascular embarrassment, but there is no proof that the fall in osmotic pressure may not, *per se*, so upset the metabolism of the cells that their protoplasm begins to disintegrate.

A negative balance was claimed by early workers, on slender evidence (Spiegler, 1901; Marriott, 1923), to follow water deprivation. It has repeatedly been shown to accompany experimental intestinal interference in animals (Whipple, Cooke, and Stearns, 1917; Haden and Orr, 1927, *a*, and Brandberg, 1929) and often attributed to toxaemia. It has been reported clinically in cases of gastric tetany (Steinitz, 1928), but the difficulty of maintaining an adequate nitrogen and calorie intake in such cases must not be overlooked. The nitrogen balance was investigated in these experiments because (Michelson, 1933) it was thought that a simple salt deficiency might make it negative, and since it did so there does not seem to be any need to invoke a toxaemia to explain the negative balance in the intestinal conditions.

### *The Percentage Loss of Sodium and Chloride*

The difficulty in estimating this lies in the uncertainty about the total amount of these elements in an adult's body. No direct determinations seem to have been made. According to Darrow and Yannett (1925) and Lavietes *et al.* (1935), there is little sodium, if any, except in the extracellular fluids which constitute 20% of the body weight. The same is true of chlorine if one excepts the red blood corpuscle. Calculating, therefore, that 1 litre of extracellular fluid contains 3·3 gm of sodium, R.A.M. contained 43 gm of sodium when the experiment began. He lost 17·5 gm, *i.e.*, 41%. Calculating the chloride in the same way R.A.M. contained 47 gm of chloride, say 50 gm making a small allowance for the chloride in the red cells. He lost 20·8 gm, *i.e.*, 41·5%. According to Winter (1934) rats contain about 0·12 % of chloride (as Cl). Rosemann (1910) whose figures were accepted by Lim and Ni (1925–26) found that adult dogs contained 0·11% of chloride and new born dogs rather more than 0·2%. Human foetuses contained between 0·197 and 0·29% and several workers quoted by Rosemann found new born infants to contain 0·146–0·189% of chloride, but adults certainly contain less. Magnus Levy (1910) suggested 0·1227% from an analysis of individual organs and Rosemann endorsed this. Assuming these figures to be true, R.A.M. would contain about 81 gm of chloride and would have lost on this basis of calculation 26% during his experiment.

The lower would seem to be the more probable answer, so that R.A.M. may be said to have lost between 25 and 30% of his extracellular ions. R.B.N.'s losses were of the same order but very slightly higher.

### *The Function of the Kidney*

A rise in the blood urea in cholera was suggested in 1832 (O'Shaughnessy, 1831–32); it was noted in diabetic coma by Colin in 1868 and in intestinal obstruction in 1914 by Tileston and Comfort. It has been extensively investigated clinically and experimentally in the last 20 years and is known to be a part of these and similar diseases (Addison's disease) in which loss of extracellular base and (or) water are prominent features. It was hoped to reproduce this rise in blood urea by simple salt deprivation and one of the main objects of the present experiments was to do so and to study its causation. Table II shows that the primary object was achieved, and the whole question has accordingly been investigated. The results are now being collected for publication, but since it is impossible to discuss them without extensive reference to the literature it is proposed to publish them in a separate paper.

Every thanks are due to Miss Edwards and R. B. Niven for their whole-hearted participation in these experiments. The author is also indebted to Miss Widdowson for a great deal of valuable help at almost every stage and to his wife and Mrs. MacGregor for their meticulous care in the preparation of the food. Professor J. B. S. Haldane has been good enough to go through the manuscript.

## SUMMARY

Forced loss of sodium and chlorine was produced by a very low NaCl intake and sweating. At least 25 to 30% of the body's extracellular ions were removed in this way. The fluid intake was not limited.

Such deprivation led to aberrations of flavour, cramps, weakness, lassitude, and severe cardio-respiratory distress on exertion.

The nitrogen balance became negative and the blood urea rose.

When subjected to such treatment the human body compromised between (*a*) maintenance of its total osmotic pressure at the expense of anhydraemia a reduction of blood volume, rise of haemoglobin, proteins and colloidal osmotic pressure in the serum, and (*b*) maintenance of its plasma and extracellular fluid volumes at the expense of a reduction in the concentrations of sodium and chloride in the serum, with a fall in its total osmotic pressure. Some evidence was obtained that (*b*) was followed by a fall in the total and colloidal O.P. of the cells.

## REFERENCES

Adolph, E. F. (1921). 'J. Physiol.,' vol. **55**, p. 114.
Beattie, F. (1928). 'Biochem. J.,' vol. **22**, p. 711.
Brandberg, R. (1929). 'Act. chir. scand.,' vol. **65**, p. 415.
Colin (1868). 'Gaz. hebd. méd. Chir. Ser. 2,' vol. **5**, p. 467.
Darrow, D. C., and Yannet, H. (1935). 'J. Clin. Invest.,' vol. **14**, p. 266.
Haden, R. L., and Orr, T. G. (1927, *a*). 'J. Exp. Med.,' vol. **45**, p. 433.
—— (1927, *b*). 'J. Exp. Med.,' vol. **46**, p. 709.
Harrison, G. A. (1930). "Chemical Methods in Clinical Medicine," 1st ed.
Hartwell and Hoguet (1912). 'J. Amer. Med. Ass.,' vol. **59**, p. 82.
Hawk, P. B. (1923). "Practical Physiological Chemistry," 8th ed.
Herrin, R. C. (1935). 'J. Biol. Chem.,' vol. **108**, p. 547.
Hubbard, R. S. (1933). 'J. Biol. Chem.,' vol. **100**, p. 557.
Kramer, B., and Tisdall, F. K. (1921). 'J. Biol. Chem.,' vol. **46**, p. 339.
Lavietes, P. H., D'Esopo, L. M., and Harrison, H. E. (1935). 'J. Clin. Invest.,' vol. **14**, p. 251.

Lim, R. K. S., and Ni, T. G. (1925–26). 'Amer. J. Physiol.,' vol. **75**, p. 475.

Loeb, R. F., Atchley, D. W., and Stahl, J. (1935). 'J. Amer. Med. Ass.,' vol. **104**, p. 2149.

McCance, R. A., and Shipp, H. L. (1931). 'Biochem. J.,' vol. **25**, pp. 449, 1845.

—— (1933). 'Special Rep. Med. Res. Coun. Lond.,' No. 187.

McCance, R. A., and Watchorn, E. (1931). 'Quart. J. Med.,' vol. **24**, p. 371.

McCance, R. A., Widdowson, E. M., and Shackleton, L. R. B. *Unpublished.*

McEwen, O. R. (1935). 'Lancet,' vol. **1**, p. 1015.

Magnus Levy, A. (1910). 'Biochem. Z.,' vol. **24**, p. 363.

Marriott, W. M. (1923). 'Physiol. Rev.,' vol. **3**, p. 275.

Michelsen, J. (1933). 'Arch. exp. Path. Pharmak.,' vol. **173**, p. 737.

Moss, K. N. (1923–24). 'Proc. Roy. Soc.,' B, vol. **95**, p. 181.

O'Shaughnessy, W. B. (1831–32). 'Lond. Med. Gaz.,' vol. **9**, p. 486.

Rosemann, R. (1910). 'Pflügers Arch.,' vol. **135**, p. 177.

Silvette, H. (1934). 'Amer. J. Physiol.,' vol. **108**, p. 535.

Silvette, H., and Britton, S. W. (1933). 'Amer. J. Physiol.,' vol. **104**, p. 399.

Spiegler, A. (1901). 'Z. Biol.,' vol. **41** (N.F., vol. **23**), p. 239.

Steinitz, H. (1928). 'Z. klin. Med.,' vol. **107**, p. 560.

Swingle, W. W., Pfiffner, L. J., Vars, H. M., and Parkins, W. M. (1934). 'Amer. J. Physiol.,' vol. **108**, p. 428.

Tileston, W., and Comfort, C. W. (1914). 'Arch. Intern. Med.,' vol. **14**, p. 620.

Van Slyke, D. D. (1923–24). 'J. Biol. Chem.,' vol. **58**, p. 523.

Whipple, G. H., Cooke, J. V., and Stearns, T. (1917). 'J. Exp. Med.,' vol. **25**, p. 479.

Widdowson, E. M., and McCance, R. A. (1935). 'Lancet,' vol. **1**, p. 1437.

Winter, K. A. (1934). 'Klin. Wschr.,' vol. **13**, p. 1454.

—— (1934). 'Z. ges. exp. Med.,' vol. **94**, p. 663.

631.461

# Short-Period Fluctuations in the Numbers of Bacterial Cells in Soil*

By Charles Bingham Taylor, Bacteriology Department, Rothamsted Experimental Station

(*Communicated by Sir John Russell, F.R.S.—Received November* 19, 1935)

## INTRODUCTION

Seasonal changes in the numbers of micro-organisms in fresh soil were first reported at the beginning of the present century; their existence has since been confirmed by workers in many different parts of the world. (For references, *see below*.) More recently, short-period fluctuations in bacterial numbers were found to exist. Such fluctuations were found in plate counts from daily samples of field soil by Cutler, Crump, and Sandon (1922) and from 2-hourly samples by Thornton and Gray (1930).

Periodic determinations of bacterial numbers in soils other than those taken from natural conditions have been few in number and have usually been made as checks on work of some other nature. In earlier work of Russell and Hutchinson (1909), soil incubated at room temperature showed fluctuations in microbial content over such a short interval as 8 hours and over as long a period as 60 days. In their later work, Russell and Hutchinson (1913) working with three soils of different moisture contents, dry, moist, and saturated, incubated at constant temperature, found changes in numbers between samples taken at from 5- to 8-day intervals. Such changes in numbers were not related to temperature, nor necessarily to moisture changes. A more intensive study was made by Allison (1917). He brought soil samples into the laboratory and made bacterial and fungal counts at short intervals of time. Samples taken during the winter showed a drop in numbers of as much as 40% during the first 1½ hours' storage, followed by a large rise after some hours; in summer the initial decrease was less pronounced, this being attributed to the fact that at that season outdoor temperatures more nearly approached indoor temperatures. Cutler and Dixon (1927) found that, with soil stored at laboratory temperatures in deep narrow bottles, bacterial numbers decreased steadily over a period of 5 weeks. In soil stored in pots with a large surface area, fluctuations in bacterial numbers of as much as 100% were obtained from week to week, and the soil in general behaved as fresh soil.

---

* This paper forms part of matter submitted in a Thesis for the Degree of Ph.D. in the University of London.

Cutler (1923) inoculated sterilized soil with suspensions of (*a*) bacteria only, (*b*) bacteria plus amoebae, and (*c*) with bacteria plus flagellates. Daily counts were made. Results showed that the more extensive bacterial fluctuations occurred in the presence of protozoa, but the run of the experiment was too short to make this result conclusive. Protozoa reduced the number of bacteria; the flagellates reduced the numbers of bacteria more powerfully than did amoebae. Telegdy-Kováts (1932) used sand instead of soil and confirmed Cutler's results as to the fall in bacterial numbers produced by protozoa.

Whilst working out the life cycle of *Bacillus radicicola*, Thornton and Gangulee (1926) noted fluctuations in numbers at 2-hourly intervals in a sterilized soil inoculated with a suspension of the organisms. Evidence showed that during the course of the life cycle, reproduction by binary fission gave way to reproduction of multiple fission, and vice versa. Consequently when the organisms reached that stage of the life cycle at which multiple fission took place, a rapid increase in numbers occurred.

It was eventually realized that plate counts represented a very small percentage of the total numbers of bacteria in soil, since no plating medium permits them all to develop into colonies. Several methods for the enumeration of total numbers of micro-organisms by direct microscopic examination were devised, most of which involve an unknown but probably large error in estimating the mass of soil upon which the count is actually made. This difficulty was avoided by the ratio method of Gray and Thornton (1928). This consists in adding a known volume of a counted suspension of indigotin particles to a given mass of the soil, preparing and staining films and counting bacteria and indigosta particles in random microscope fields. A ratio of bacteria to indigotin particles is thus obtained, and from the knowledge of the absolute concentration of indigotin particles in the soil, the total numbers of bacteria are calculated. The history of the direct count technique and a critical test of the ratio method is given in the paper of Thornton and Gray (1934).

A comparatively small amount of work has been performed on the total bacterial numbers of soil, but Conn (1918), Joffe and Conn (1923), Thatcher and Conn (1927), Conn and Darrow (1930), Germanov (1930), Paulie (1934), and Richter and Richter (1925), using direct methods other than the ratio method, have either made total bacterial counts or determined the relative abundance of bacteria. Vandecaveye and Villanueva (1934) determined numbers by the Conn method in four soils of different manurial treatments incubated at room temperature, and classified the organisms as cocci and rods. Short-time changes were noted but the low total numbers obtained (40–60 millions per gram) suggest that their counts were erroneous. Indeed, results obtained by methods developed prior to the indigo technique are suspect owing to our ignorance of the accuracy of such methods. Important sources of error are discussed by Thornton and Gray (1934).

In view of the limited information provided by plate counts in soil and the somewhat contradictory results obtained by previous workers who used the plating method, it seemed desirable to investigate more fully the reported short-time changes in numbers of micro-organisms in a soil plot. It was decided to count bacteria primarily by the microscope ratio method to determine the total population and to use the plate method for comparison. It was further decided to study the behaviour of bacteria in soil under controlled laboratory conditions in the hope of analysing the cause of fluctuations found in the field.

The work to be described was divided into three sections which followed logically on the experimental results as they progressed (1) counts were made first at daily and subsequently at 2-hourly intervals in soil taken from a garden plot; (2) daily counts were made from soil incubated at constant conditions of moisture and temperature; (3) daily counts were made from sterilized soil inoculated with pure or mixed cultures and also incubated at constant conditions.

## TECHNIQUE

The technique used for the plate count method was the standard method used at Rothamsted (Thornton, 1922), with a modification that the dilutions were made up exactly into sterile flasks from a bulk of sterile diluting liquid; this avoided the difficulty that the dilutions diminished in volume in varying amounts during sterilization. The media used throughout consisted of mannite-salts (called " count ") and/or soil extract agar. The " count " medium was developed (Thornton, 1922) with the special object of repressing spreading colonies: the accuracy of results obtained with it has been extensively tested by statistical methods (Fisher, Thornton, and Mackenzie, 1922). The soil extract was made up according to the method of Löhnis (1913), and from soil of the same plot as that from which the present bacterial counts were made. In view of the fact that the composition of such an extract might vary from time to time, a sufficient initial quantity was made up to last throughout an experiment. Plates were incubated at 21° C for a period which varied with the type of experiment: 10 days for fresh and incubated soils, and 5 to 8 days for sterilized inoculated soils, depending on the rapidity of growth of the added organisms.

The method used for counting the total numbers of micro-organisms was the ratio method due to Gray and Thornton (1928) and Thornton and Gray (1934). A few modifications of their methods were introduced. As soon as the drops of soil suspension *plus* indigotin had been made, the slides were introduced into a moist chamber for a period of 10 minutes. This stay in the chamber ensured the even distribution of bacteria and indigotin particles, which, when the slides were not so treated, had a tendency to concentrate at the edge of the drop. The bacteria and indigotin particles were counted in ten random fields in each drop. Usually eight drops were examined—four from each of two duplicate slides. In all, the bacteria and indigotin particles were counted in 80 microscope fields for each soil sample: that is, between 500 and 1000 particles, with a similar number of bacteria. For perfect performance the method entails considerable practice, especially in the recognition of the stained organisms (the size of which may reach the limit of vision) and their differentiation from occasional stained pieces of organic material. A high efficiency, with surprisingly good agreement between duplicate samples, can be reached after some experience.*

Owing to the difficulty in differentiating between bacteria and actinomycetes, the latter have been included in the figures for bacteria in the ensuing experiments.

## I—COUNTS OF MICRO-ORGANISMS IN A GARDEN PLOT

*Experiment A I. April* 23, 1934—The first two experiments here described were made with the object of determining whether the daily fluctuations previously found with plate counts would also appear in the total cell counts, and, if so, whether any relationship existed between the numbers obtained by the two methods.

The plot on which experiments were carried out was the same as that used by Thornton and Gray (1930), which has remained fallow and untreated since their work. The plot was divided into halves, and each half into 4 feet by 3 feet rectangular sections.

Samples were taken at 9.30 a.m. each morning, using a 2-inch diameter cylindrical steel sampler. The top inch of the soil was discarded, and a core to the depth of 6 inches was taken at random from each of the six sections of a half-plot and these cores were mixed to form a composite sample. The process was repeated for the second half-plot. The two composite samples were brought into the laboratory at once, and each was thoroughly mixed and sifted through a sieve having circular holes of 3 mm diameter. From each of the two samples seven plates were poured on both count and soil extract media. Slides for the microscope counts were also prepared from each sample. Moisture determinations were made by weighing the soils before and after heating at 105° C for 24 hours. The experiment was continued for 10 successive days, during which time 1·24 inches of rain fell. Fig. 1, *a*, shows the numbers of organisms found in the separate half-plots, and fig. 1, *b*, the means of the two half-plots, compared with the soil moisture content.

---

* Samples of indigotin from different manufacturers vary considerably. It is ssential to use a brand which will provide a good suspension of particles.

Considerable changes in bacterial numbers were recorded by both plate and microscope counts, fig. 1, *a* and 1, *b*. All numbers showed a very large drop on the sixth day followed by a subsequent increase. With the total count, this rise amounted to approximately 1000 millions of organisms per gram of soil during the 2 days. Numbers obtained on soil extract agar were almost twice as high as those obtained on count agar, but these numbers represented only 1·1 and 1·9% respectively, of total numbers as determined by the ratio method. The curves all show a general resemblance although maxima were recorded on different days on the two media.

A *z* test (Fisher, 1932) was made to compare the variance between mean counts obtained at different times with variance between simultaneous half-plot samples, in order to find whether the changes in numbers in time significantly exceeded differences ascribable to soil heterogeneity. Correlation coefficients were also calculated.

Table I shows the values obtained.

The changes in bacterial numbers in time significantly exceed the variance estimated from simultaneous half-plot samples, and give values of *z* having with all three methods a probability of less than 5%, and, with two of them (the count medium and microscope counts) a probability of less than 1%.

A positive correlation was shown between plate count results and moisture, and also between the total count figures and rainfall.

*Experiment A II. June* 6, 1934—This experiment, though essentially a repetition of experiment *A I*, took place under very different weather conditions. Only 0·32 inches of rain had fallen in the 4 weeks previous to the commencement of the experiment, and only 0·09 inches fell during the samplings. The experimental detail was the same as that used in

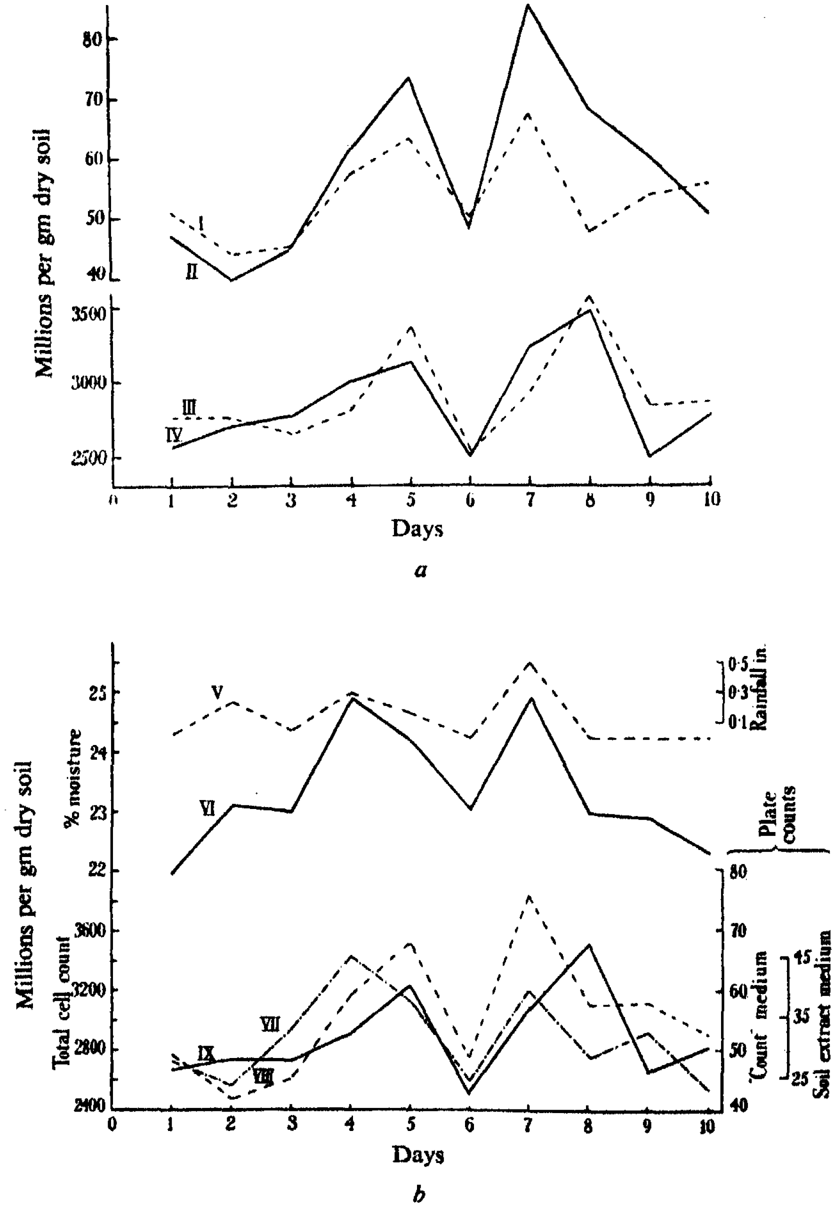


Fig. 1—Bacterial counts from daily plot samples. *a*, separate half-plot counts, I, II, soil extract plate count; III, IV, total bacterial cells; *b*, means of two half-plots, V, rainfall; VI, moisture; VII, count medium plates; VIII, soil extract plates; IX, total bacterial cells.

experiment *I*. Work was discontinued after the eighth day, but a heavy rain of almost 0·75 inches fell on the following day and presented an admirable opportunity to study the effect of a sudden increase in soil moisture content, so the experiment was re-started on the eleventh day and continued over nine further successive days.

TABLE I—EXPERIMENT *A I*. DAILY SAMPLINGS FROM GARDEN PLOT

Analysis of variance in bacterial numbers. Comparisons of fluctuations in time with variance between half-plots

| | Value of $z$ |
|---|---|
| Soil extract agar: .......... | 0·7369 |
| Count agar: .............. | 1·0833 |
| Total cell count: .......... | 1·1554 |

Where $z = 0{\cdot}81$,
$P = 0{\cdot}01$.

Where $z = 0{\cdot}56$,
$P = 0{\cdot}05$.

*Correlation Coefficients*

| Between: | $r =$ | P | |
|---|---|---|---|
| Total count and platings on " count " medium ...................... | 0·3419 | >0·1 | Not significant |
| Total count and platings on soil extract medium ................ | 0·5947 | >0·05 | Not significant |
| Total count and moisture .......... | 0·3953 | >0·1 | Not significant |
| Total count and rainfall .......... | 0·7110 | 0·02 | Significant |
| Platings on soil extract medium and rainfall ....................... | 0·583 | >0·05 | Not significant |
| Platings on " count " agar and rainfall .......................... | 0·635 | <0·05 | Significant |
| Platings on soil extract agar and moisture ...................... | 0·6851 | <0·05 | Significant |
| Platings on " count " agar and moisture ...................... | 0·8289 | <0·01 | Significant |
| Platings on " count " agar and platings on soil extract agar ............ | 0·6712 | <0·05 | Significant |
| Rainfall and moisture ............ | 0·7230 | <0·02 | Significant |

Previous to the rain, total numbers of micro-organisms fluctuated in a remarkable manner, fig. 2, maintaining a pronounced diurnal rhythm the alternate day figures showing a progressive decrease until the final day, when numbers were less than a half of their original figures. The heavy rainfall caused no immediate response and not until the fourth day was any increase noticed. On the fifth day, a rise in total bacterial numbers of some 1700 millions per gram took place, followed by a steady drop over a 3-day period, with another large rise during the last 2 days. Numbers obtained by the plate method in the first half of the experiment did not show the striking diurnal rhythm found in the total counts. After the rain, numbers found with soil extract agar had increased considerably by the third day, but the numbers obtained with count medium showed no immediate rise. A similarity is noticeable between the results on the two types of media before the rain, but afterwards the results were discordant.

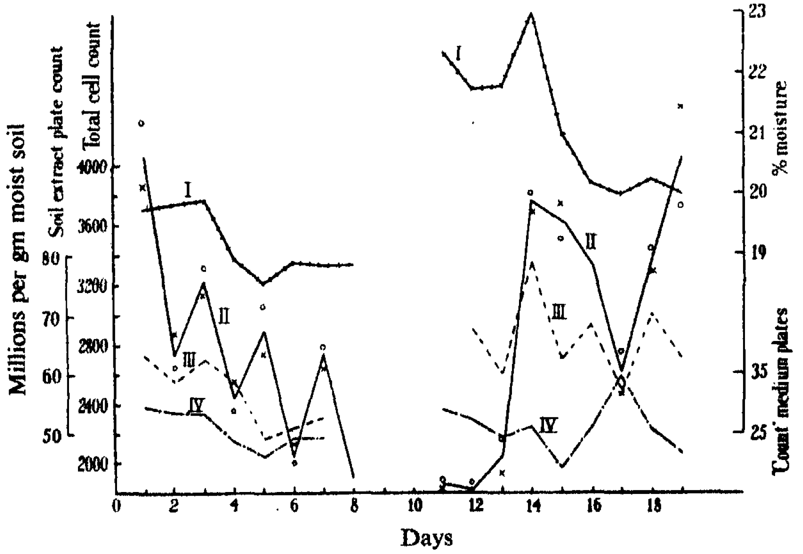


FIG. 2—Bacterial counts from daily plot samples commenced June 6, 1934. — total count; -- count medium; — · — soil extract medium; -ᐩᐩ- moisture. The crosses and circles above and below the curve for total bacterial cells in this figure represent the determinations from individual half-plots. They have a similar significance in subsequent figures.

Very significant values of $z$ were obtained when the fluctuations in total numbers were compared with the variance between half-plots, Table II, but this did not hold for the numbers obtained by plate counts. Since many of the numbers of $\chi^2$ of replicate plates were excessive, this discordance was ascribed to the plate method itself rather than to true differences in bacterial population between half-plots. Although there appeared to be a general resemblance between total numbers and soil moisture in the post-rain period, there was no significant correlation.

As the plate count fluctuations were not significant, no correlations can be made with moisture or temperature.

TABLE II—EXPERIMENT *A II*

Comparisons of fluctuations with the variance between half-plots

| | $z =$ | P |
|---|---|---|
| Total count before rain | 1·8008 | $<0{\cdot}01$ |
| Total count after rain | 0·9568 | $<0{\cdot}01$ |
| Count media before rain | 0·2598 | $>0{\cdot}05$ |
| Count media after rain | 0·1989 | $>0{\cdot}05$ |
| Soil extract media before rain | 0·3923 | $>0{\cdot}05$ |
| Soil extract media after rain | 0·5217 | $>0{\cdot}05$ |

*Correlation*

Total count after rain, and moisture........ $r = 0{\cdot}3542$ Not significant

### *Counts of Micro-Organisms at 2-hourly Periods*

*Experiment A III. September* 24–26, 1934—The next three experiments were made to see whether fluctuations in total bacterial numbers similar to those found from daily samples could be detected between samples taken at 2-hourly intervals, and if so whether these counts, made at shorter intervals, would show any relation to the plate counts or to moisture or temperature.

The plan of the experiment was similar to the two previous experiments except that samples were taken at 2-hourly instead of daily intervals. One medium, soil extract, was used throughout, and 10 replicate plates were poured in place of the usual seven.

Owing to the length of time over which the 2-hourly samplings were taken it was necessary to employ a team of workers in this experiment. Four workers (J. E. Rudorf, H. G. Thornton, Hugh Nicol, and the author) took part in the work, two operating simultaneously in 8-hour shifts. Of these two, one worker dealt with one half-plot sample, pouring plates and making preparations for the ratio count therefrom. After incubation the colonies on all the plates from half-plot A were counted by one worker (H. G. Thornton) and all from half-plot B by a second worker (J. E. Rudorf). The whole of the microscope counts from the prepared slides were made by the author.

Large fluctuations in total numbers of bacteria occurred, fig. 3, over the 48 hours, independently of the time of day or night. A large rise between 11 a.m. and 1 p.m. followed early morning rain showers, but the subsequent drop, and rise between 5 p.m. and 9 p.m. could not similarly be explained. A comparison of the fluctuations with the variance between half-plots showed that these were very significant. A value of $z = 1 \cdot 6389$ was obtained, which has a probability of less than 1%. Detailed counts of bacteria in the half-plots are shown in Table X.

Plate counts showed significant fluctuations ($z = 1 \cdot 4778$; $P < 0 \cdot 01$) which were entirely independent of total numbers. Maxima and minima were not observed at any regular time of the day or night. No significant correlation was obtained between bacterial numbers (as determined by either method) and moisture, temperature, or barometric pressure.

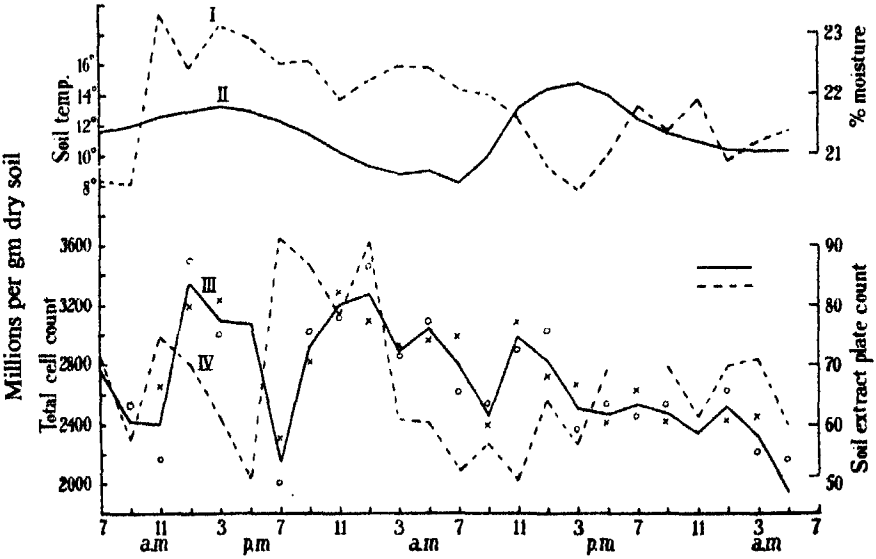


FIG. 3—Bacterial counts from 2-hourly plot samples commenced September 24, 1934. I, moisture %; II, temperature; III, total cell count; IV, plate count.

*Experiment A IV. November* 6, 1934—In all previous work, the time which elapsed between sampling and the commencement of the plating, did not exceed 15 minutes, but it seemed of importance to determine whether any rapid change took place after removal of the soil sample from the plot. To test this point, a slight modification was made in the usual procedure; separate sampling was not made of the two plot halves, but on each occasion a composite sample was made of two cores taken at random from each of six equal sections. Plating and direct count slides were made from the sifted sample at once, and also after a period of 30 minutes, during which the soil had lain on the bench at room temperature. Thus the effect, if any, of 30 minutes' storage could be determined. Seven successive samplings were made at 2-hourly intervals from 10 a.m. to 10 p.m.

Results obtained with the microscope count were surprising, insomuch as there were no rapid and large fluctuations, fig. 4, the numbers declining almost steadily during the day except for a slight rise at 4 p.m. However, on soil extract agar, plate count numbers showed striking changes during the entire day, a rise and fall of some 50% being obtained between 2 p.m. and 6 p.m. No effect is ascribable to variation in moisture content, since the differences between moisture contents did not exceed 0·3%. In fig. 4 the graphs represent mean counts; crosses, counts of individual samples before, and circles, after 30 minutes' storage. This storage clearly produced no significant effect on the counts of micro-organisms.

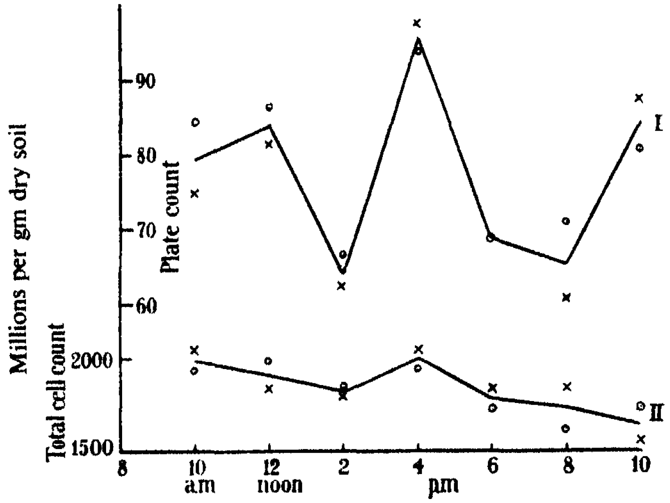


FIG. 4—Bacterial counts from 2-hourly plot samples, November 6, 1934. I, plate count; II, total cell count.

Calculated for the plate counts, $z$ had the significant value 1·2455, having a probability of less than 1%.

*Experiment A V. March* 15, 1935—In view of the relation found by Cutler, Crump, and Sandon (1922) between daily plate counts of bacteria and dilution counts of protozoa, counts were made to ascertain whether numbers of protozoa fluctuate at periods of less than 1 day, and if so, whether such fluctuations are related to those of the bacteria. The method of Cutler (1920) for the determination of numbers of active and total protozoa calls for 30 dilutions and 60 Petri dishes per sample. This procedure could not be carried out by one person every 2 hours. A preliminary count was therefore made on soil taken from the plot and the approximate protozoal numbers determined; dilutions—four in all—on either side of this figure were arranged so that any changes would be detected.

The data given by Thornton and Gray (1930) suggest the existence of short-time changes in soil nitrate, but were somewhat inconclusive on this point. Further evidence seemed desirable, and determinations of soil nitrogen as ammonia and as nitrate were therefore made by Mr. J. B. Marshall. Total bacterial numbers were estimated and plate counts made on count and soil extract media.

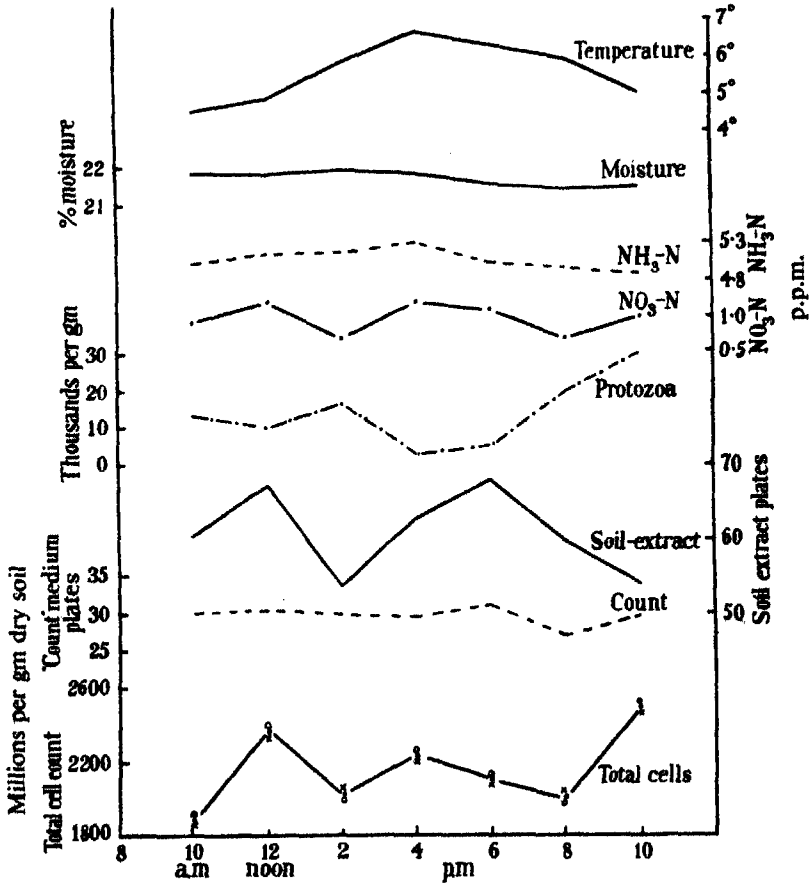


Fig. 5—Data from 2-hourly plot samples, March 15, 1935.

In contrast to experiment *A IV*, total bacterial numbers showed a highly significant series of fluctuations over the 12 hours, fig. 5, a comparison of these fluctuations with half-plot variances giving $z = 2 \cdot 8619$ ($P < 0 \cdot 01$). The plate count on count agar yielded almost the same figures throughout; fluctuations were, however, obtained with soil extract media but were not significant, owing to the divergence between duplicate samples. Figures for the nitrate content were low and the amounts varied very little from time to time. Such variations may not be statistically significant although very close agreement between parallel samples was obtained, Table III. It is of interest to notice moreover that the nitrate curve obtained oscillates in sympathy with the curve of the total bacterial numbers. Ammoniacal nitrogen content remained almost constant throughout the day. Total protozoal numbers showed strong evidence of time changes. Unfortunately the use of so few dilutions created a very high standard error, and except for the drop from 16,000 to 3000 protozoa per gram between 2 p.m. and 4 p.m., and the subsequent rise from 3000 to 30,000 per gram, no intermediate changes were significant.

TABLE III—AMMONIACAL AND NITRATE NITROGEN, PROTOZOAL AND BACTERIAL (SOIL EXTRACT MEDIA) NUMBERS AT 2-HOURLY INTERVALS IN THE GARDEN PLOT. MARCH 15, 1935

| | N in p.p.m. (duplicate samples) | | Protozoa per gram | Bacteria millions per gram, microscope counts |
|---|---|---|---|---|
| | $NH_3$ | $NO_3$ | | |
| 10 a.m. …….. | 5·0 | 0·9 | 13,000 | 1873 |
| | 5·0 | 0·9 | | 1893 |
| 12 noon …….. | 5·1 | 1·2 | 9,400 | 2395 |
| | 5·1 | 1·2 | | 2360 |
| 2 p.m. ………. | 5·3 | 0·8 | 16,000 | 2006 |
| | 5·0 | 0·6 | | 2024 |
| 4 p.m. ………. | 5·3 | 1·2 | 2,900 | 2258 |
| | 5·3 | 1·2 | | 2225 |
| 6 p.m. ………. | 5·0 | 1·1 | 4,900 | 2129 |
| | 5·1 | 1·1 | | 2067 |
| 8 p.m. ………. | 5·0 | 0·8 | 19,000 | 1984 |
| | 5·0 | 0·6 | | 2012 |
| 10 p.m. ………. | 5·0 | 1·0 | 30,000 | 2501 |
| | 4·8 | 1·0 | | 2510 |

The curve for protozoal numbers runs inversely to the curve for total bacteria, save for the last point, so that a relationship is suggested, although the accuracy of the protozoal counts does not justify statistical treatment. Temperature and moisture were not correlated with either bacterial or protozoal numbers.

## II—EXPERIMENTS ON INCUBATED SOIL

In the work above described and in that of other workers, soil temperature and moisture were sometimes found to be correlated with fluctuations in bacterial numbers and sometimes not. The following experiments were made in order to determine whether fluctuations would occur when the factors of temperature and moisture changes were eliminated by keeping soil as far as possible at constant temperature and moisture in the laboratory.

Soil from the garden plot was brought into the laboratory and sifted as before through a 3-mm sieve. Portions of 30 gm were weighed out and placed in Petri dish lids in order to obtain a very large ratio of surface area to depth, as advocated by Cutler and Dixon (1927). The Petri dish lids were stacked on a circular tin base under a bell jar, a dish of water being included to saturate the atmosphere. The bell jar was placed in an incubator at 22° C. Despite the theoretically saturated atmosphere, the soil sample slowly lost moisture throughout the experiment. The loss was, however, quite steady. A whole dish of soil was regarded as a sample, separate counts being obtained each day from two separate dishes.

In preliminary work, bacterial numbers at first increased in a manner suggesting a growth curve until a maximum was reached on the seventh day; in order to avoid recording intrusive effects of this phenomenon, soil samples were incubated for at least 7 days before commencing the counting.

*Experiment B I*—Bacterial counts were made by both the direct method and the plate method, using count medium. Counts were begun on the ninth day after the commencement of incubation, and continued daily for 10 days. The results are shown in fig. 6.

Significant fluctuations in total numbers of bacteria took place over the 10-day period. The comparison of fluctuations with variance between simultaneous samples gave a value of $z = 0 \cdot 8201$ which was a probability of less than 1%. No significant changes in plate counts were found, differences between duplicate samples being as large as day-to-day mean differences.

*Experiment B II*—The same technique was used as in the previous experiment except that the duration was extended to 18 successive days. Similar results were obtained, there being significant fluctuations in total numbers and non-significant plate counts fluctuations throughout. Results are shown in fig. 7.

*Experiment B III*—In this experiment the effect of a temperature change shortly before the beginning of the experiment was studied. Eight consecutive daily samples were taken.

The samples were incubated at room temperature for 6 days, and placed in a 22° C incubator only 24 hours before the commencement of sampling. Total counts were made and also plate counts on both media. An enormous change in total numbers took place between the third and sixth day of sampling, fig. 8. Numbers doubled between the third and fourth day, and returned to their original state by the sixth day. For the first time, changes obtained with plate counts were found significant for both media. Fluctuations were similar in direction, except that the initial maximum appeared a day earlier on the soil extract medium.

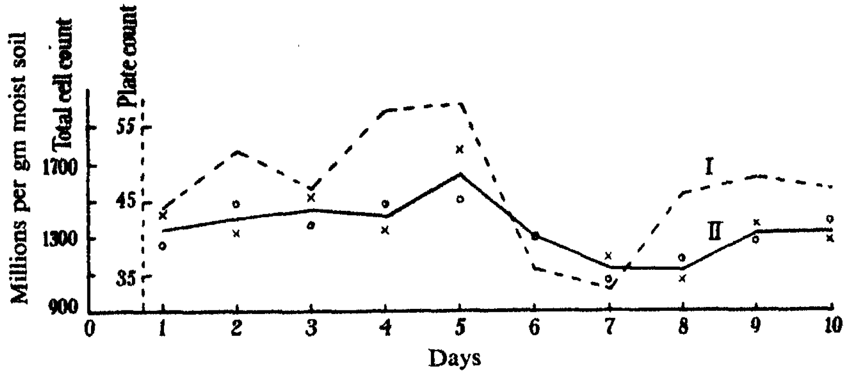


FIG. 6—Bacterial counts at daily intervals from soil incubated at 22° C. – – – plate count; — total bacterial cells.

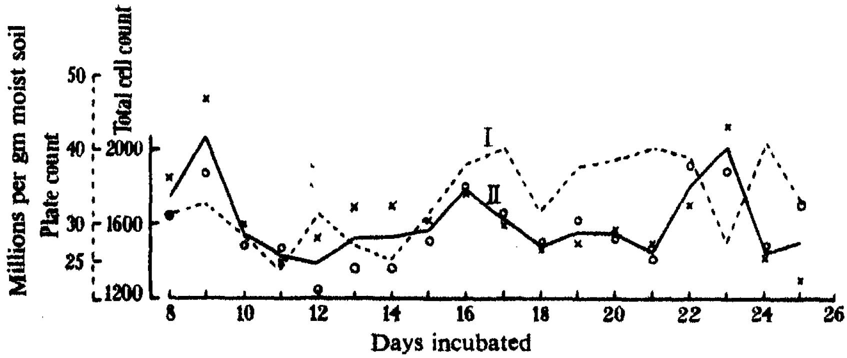


FIG. 7—Bacterial counts at daily intervals from soil incubated at 22° C. – – – plate counts; — total bacterial cells.

The plate count curves suggest that the large initial increase was due to the placing of the soil in the incubator, with a consequent increase in temperature during a short space of time before sampling. This temperature change probably caused the burst of activity recorded by the total cell count.

The rise in total numbers took place after the rise in the plate count. The same tendency was also noticeable in the garden plot experiment *A I* and in the incubated soil experiment *B I*. The data are not sufficient for the significance of this phenomenon to be judged.

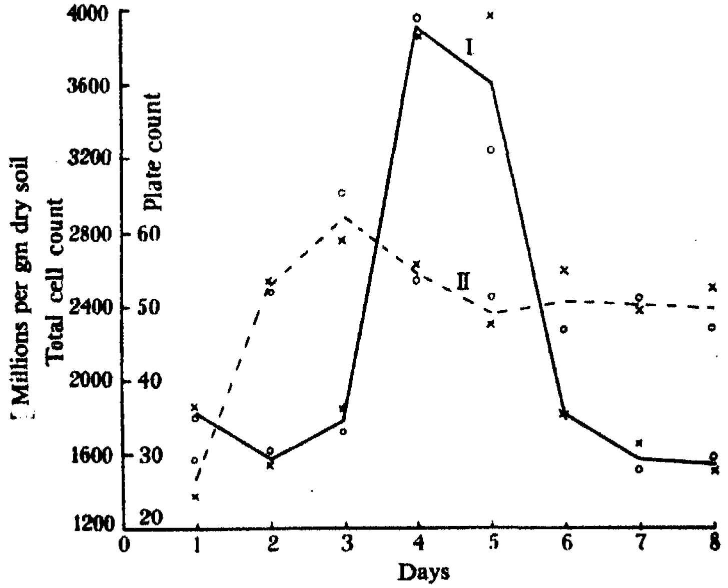


FIG. 8—Bacterial counts from soil incubated at 22° C. – – – plate count; — total bacterial cells.

The results of the three experiments last described show that fluctuations in bacterial numbers can occur even when the moisture and temperature conditions of the soil were kept relatively constant.

*Experiment B IV*—To compare numbers obtained from soils kept at different temperatures, the experimental technique was changed considerably. Soil from the same plot as before was sifted through a 1-inch riddle, and four large earthenware pots, which held approximately 30 kilos each, were filled with the coarsely sifted soil.

The four pots were placed in an unheated greenhouse, and, all having been left there for a period of 6 weeks, two pots were removed and placed in a heated greenhouse, the temperature of which was 16° C—varying less than 2° C from day to day. The other two pots remained in the unheated greenhouse, the temperature of which was not controlled and averaged only 5° C at the time of sampling, March 15. After the pots had remained in their new environment for a period of 7 days, composite samples were taken from each pot by means of a cork borer. The soils were then sifted through a 3-mm sieve, and, immediately after this sieving, counts of bacteria were made by the microscope and plate methods. Moisture contents of the soils showed very small day-to-day differences.

Counts of total numbers of micro-organisms showed no significant day-to-day variations but the plate counts exhibited some changes, fig. 9. Samples taken from the cold greenhouse showed fluctuations which, owing to the somewhat large differences between counts from duplicate pots, were not statistically significant ($z = 0{\cdot}3430$). These differences are shown by the circles and crosses in the graph, fig. 9. The fluctuations observed in the bacterial numbers in soil from the warm greenhouse were significant and gave a value of $z = 0{\cdot}8898$ having a probability of less than 5%. There was no parallelism in the direction of fluctuations in the two soils.

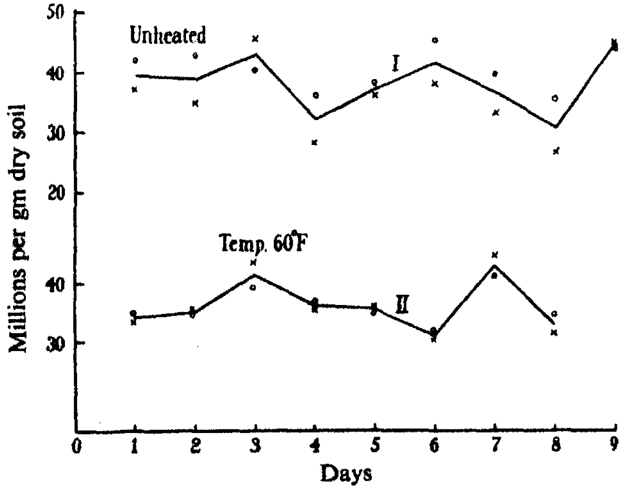


FIG. 9—Plate counts from soil stored in pots in a heated and unheated greenhouse. I, unheated; II, heated to 60° F.

A difference of some 10° C in storage temperature thus made surprisingly little differences in the mean microscope and plate count numbers, Table IV.

TABLE IV—TEMPERATURE, MOISTURE, AND BACTERIAL COUNT AVERAGES IN EXPERIMENT *B IV*

| | Temperature °C | Moisture % | Total cell count | Plate count millions per gm |
|---|---|---|---|---|
| Warm greenhouse .. | 15·7 | 18·78 | 2375 | 36·04 |
| Cold greenhouse.... | 5·2 | 20·21 | 2254 | 38·17 |

## III—EXPERIMENTS ON INOCULATED STERILIZED SOIL

The experiments described in the last section showed that the bacterial numbers in soil could fluctuate even when the soil was kept at relatively uniform moisture and temperature conditions. Those factors are therefore not the only cause of fluctuations. The soil used was fresh and contained a large diversity of competing organisms, including protozoa, suspected of being concerned in the fluctuations. Therefore, to study the behaviour of a simplified population, sterilized soil was inoculated with a pure and with mixed cultures of organisms, and thereafter periodic counts were made.

*Experiment C I*—Fresh soil from the garden plot was dried in a drying room at 100° F for 24 hours and then sifted through a 2-mm sieve. It was next spread out in thin layers in Petri dishes of 20 cm diameter and autoclaved at 15 lb pressure for 1 hour, on three successive days. This treatment completely sterilized the soil, as was shown by the absence of growth after a portion had been inoculated into nutrient medium.

A suspension in distilled water was made up of five organisms previously isolated from plot soil; these consisted of a small coccoid rod, two types of short rod, and two types of long spore-forming rods. Enough of this suspension to give the soil a moisture content of approximately 15% by weight was added drop by drop with constant mixing, and after allowing the soil to stand for 2 hours a further mixing was made. The soil was then weighed out into Petri dishes and incubated in a " saturated " atmosphere at constant temperature as in previous experiments. After 7 days' incubation, daily total and plate counts were made from duplicate samples from each of the two separate dishes. The curves of the results, shown in fig. 10, thus represent the means of four determinations.

A comparison of the fluctuations in the total count with variance between the four parallel determinations gave a value of $z = 1 \cdot 2644$, which has a probability of less than 1%. The plate counts were so large as to necessitate the use of 10 million-fold dilutions, and gave so high a variance between replicate counts that no information was gained by this method.

The fluctuations in total count thus found to exist in the soil population consisting wholly of different species of bacteria suggest that a fluctuating equilibrium may exist amongst the bacteria themselves apart from any effect from protozoa and other forms. In this experiment, however, a possible alternative explanation may be afforded by the fact that two of the bacterial types added were spore-forming. Since spores do not usually stain with erythrosin as here used, the fluctuations observed may have been due to spore formation and germination—possibly unaccompanied by any marked changes in the number of cells. The behaviour of a spore-forming type when added in pure culture to soil was therefore investigated.

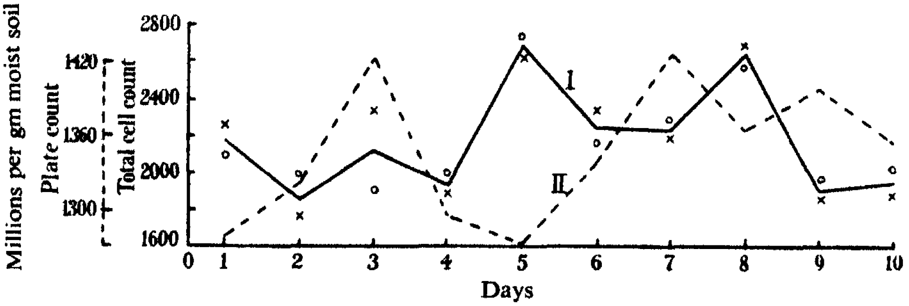


FIG. 10—Counts from a mixed culture of bacteria added to a sterilized soil. – – – plate count; — total bacterial cells.

*Experiment C III*—A similar procedure was adopted for this experiment as for the two previous ones, except that a pure culture of a long thin spore-forming rod was used, and the inoculation was sprayed on to the soil by an "atomizer" to ensure even distribution. The organism was a very prolific grower, and high counts were obtained; but it tended to "clump." Unfortunately, 2 days' plate counts were lost through use of too low a dilution. Large and significant microscope count fluctuations occurred, fig. 11, giving a value of $z = 1 \cdot 2900$ which has a probability of less than 1%. The fluctuations shown by the plate count were not significant, possibly owing to high variance between replicates.

During the microscope counting, the interesting observation was made that although the organism was in pure culture, many different shapes were to be seen such as long thick and thin rods, sporulate (swollen) rods and small coccoid shapes. Colonies appearing on plates all showed the pale yellow colour characteristic of the organisms and thus gave no evidence that contaminants were present. Vandecaveye and Villanueva (1934) and Paulie (1934) separated bacterial forms seen in counts into cocci and rods, and were under the impression that their cocci and rods represented different groups. The fact that a pure culture may give rise to many different cell shapes indicates that such claims may be entirely invalid.

*Experiment C IV*—Since significant fluctuations were found when a spore-forming organism was grown in soil, it was decided to test the effect of adding two non-sporing bacteria to sterile soil, to study their associated growth while avoiding the complications due to spore formation. In order to follow more easily the relative changes in the population of a mixed culture, a mixture of two readily distinguishable types of bacteria was added to sterilized soil. The two organisms consisted of an unidentified organism isolated from milk, and *Chromobacterium violaceum* (Schroeter) Bergonzini, this latter being included in the hope that the deep blue colonies appearing on the plate could be recorded separately. The milk organism depressed the growth of the other, and appeared alone on the plates; consequently no separate recordings of *Chromobacterium violaceum* were made. The preponderance of the milk organism nevertheless was not detrimental to counting, for it possessed the good qualities of being easily distinguishable and having no tendency to clump.

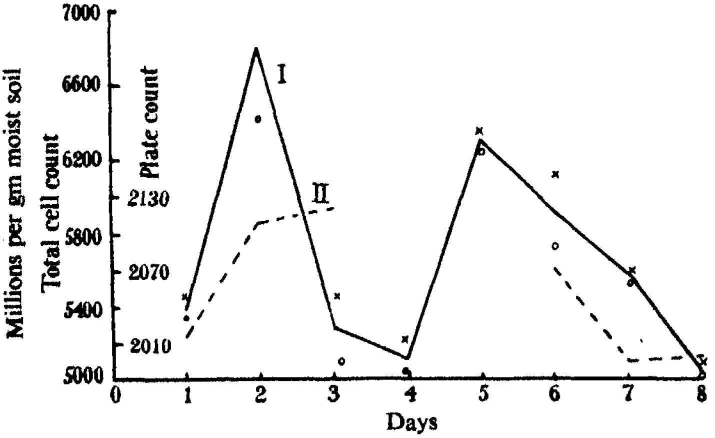


FIG. 11—Counts from a pure culture of a spore-forming organism growing in sterilized soil. – – – plate count; — total bacterial cells.

In this experiment, fig. 12, no significant fluctuations were obtained, by either method of counting, although by the ratio method, parallel examples agreed remarkably closely.

## BACTERIAL NUMBERS AT DIFFERENT TIMES OF THE YEAR

A voluminous amount of work has been performed on the seasonal variations of bacterial numbers in soil and the results have been extremely controversial. Thus Remy (1902), Engberding (1909), Conn (1910), Woitkiewicz (1914), Russell and Appleyard (1917), Brown and Halversen (1919), Lochhead (1924), Cobb (1932), and Eggleton (1934) found maxima in the spring between February and April, with additional maxima in September to October, in some cases. Hiltner and Störmer (1903) found maxima numbers in May and June, Fabricius and Feilitzen (1905) in July and August, Kruger and Heinze (1907) in July, and Newton (1930) in May and August. Fehér (1929, 1930, and 1934) found increasing numbers from April to November, and Cutler, Crump, and Sandon (1922) recorded lowest numbers in February and highest in November. Waksman (1916) obtained separate maxima for each of the four plots which he sampled, whilst Corbet (1934) and Jensen (1934) obtained fluctuations throughout the year without any definite seasonal maxima.

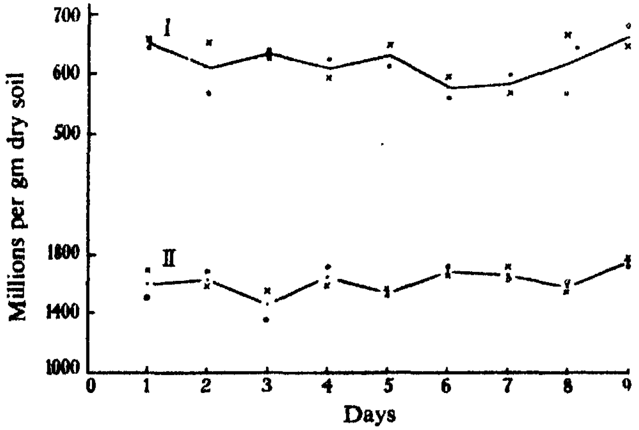


FIG. 12—Counts from a mixture of two non-sporing bacteria added to sterilized soil. I, plate count; II, total cell count.

These workers used a variety of media and techniques. Moreover, since results were taken in some instances from only one sample of soil per season or per month, it is not surprising that confusion exists.

A comparison of the bacterial numbers obtained at different times of the year in the course of the present work is shown in Table IX.

The figures represent the means from a number of samplings throughout each experiment made either at daily or 2-hourly intervals. The table also shows the numbers of separate soil samples from which each mean count is derived.

Although the figures do not cover the winter months they are interesting. Thus, the warm month of June gave no increase of bacteria over the colder months of March on count media, and the three occasions showed little difference. Upon soil extract media, larger numbers were obtained during November than during the spring and summer. The total numbers, however, behaved as might be expected, showing a rise from spring to the summer, and a decrease during the autumn.

TABLE IX—BACTERIAL NUMBERS AT DIFFERENT TIMES OF THE YEAR, AVERAGE NUMBERS IN MILLIONS PER GRAM OF SOIL

| Date | Count medium | Number of samples examined | Soil extract medium | Number of samples examined | Total count | Number of samples examined |
|---|---|---|---|---|---|---|
| March 15, 1935 | 29·6 | 14 | 61·0 | 14 | 2159 | 14 |
| April 23 to May 2, 1934 ...... | 32·1 | 20 | 55·7 | 20 | 2889 | 20 |
| June 6 to July 4, 1934 ........ | 25·8 | 34 | 66·9 | 32 | 2854 | 34 |
| September 24 to 26, 1934 .... | — | — | 66·4 | 48 | 2696 | 48 |
| November 6, 1934 ........ | — | — | 77·5 | 14 | 1845 | 14 |

## DISCUSSION

It is necessary to recognize that different species of organisms are counted by the plate and the ratio methods, and the accuracy obtained by those methods. The ratio method for the total count depends upon the staining of the organisms by the dye employed, carbol-erythrosin. Thus, live and dead cells will both be stained, probably together with an intermediate type of cell which is in a " resting " state and is incapable of reproduction. Although carbol-erythrosin has wide staining powers, certain groups of organisms and spores may not take the stain and may thus fail to be recorded. Furthermore, some actinomycete cells are indistinguishable from bacteria and are therefore included in the total count.

Numbers obtained by the plate method depend on the medium used and the ability of the organisms to use the energy sources contained therein. In the present work, numbers usually twice as high were obtained on soil extract medium as on the " count " medium, and fluctuations obtained on one of these media have often passed unrecorded by the other. Moreover, with the plate method, disagreement between parallel plates frequently vitiated the results. The much greater consistency of results obtained by the ratio method can be seen from the numbers of occasions when this method enabled significant fluctuations to be detected, whose existence could not be established by the plate method, solely owing to disagreement between parallel counts. A comparison between the numbers found by the total cell count and the soil extract plate count where bacteria, known to be capable of growth on the medium, were added to sterile soil, indicates that from 36 to 61% of the cells found by the former methods are viable.

Since rainfall conditions, the volume of soil moisture, in which the organisms proliferate, and the amount of dissolved energy forming material and dissolved oxygen, a general relationship between rainfall or moisture and bacterial numbers might be expected. Actually a correlation of such a nature was found on one occasion only, when heavy showers might have been a limiting factor. A similar isolated case was recorded by Thornton and Gray (1930) in a series of plate counts from daily samples of Broadbalk field soil, taken in showery weather. On the other hand significant changes have been observed when the moisture content has remained constant. However, the moisture content of the soil under experimentation was fairly constant, whereas some of the soils of high organic matter content, studied by other workers, varied greatly in wetness.

Normal changes in soil temperature have not had any apparent relationship with, or effect upon, bacterial numbers. However, a steady increase in numbers was observed during the first 7 days' incubation of a soil sample, and a spasmodic rise and fall in numbers was obtained by suddenly increasing the incubation temperature. No effect of light or of barometric pressure has been observed during the course of the work. Thus, no one external condition appears to be responsible for the changes observed in the soil population.

In the soil, bacteria, protozoa, actinomycetes, fungi, and algae are all competing for a limited amount of energy-forming material, and any increase in one group must be at the expense of another. There may well be an unstable equilibrium between the competing groups of the micro-population. An inverse relationship between numbers of amoebae and bacteria found by Cutler, Crump, and Sanson (1922) was by them attributed to the amoebae proliferating at the expense of the bacteria; the former group were thought to be the primary cause of the fluctuations in bacterial numbers obtained. Some evidence of such an inverse relationship between bacteria and protozoa was noted in one of the experiments, *A V*, in this work. A considerable amount of work has been done on the feeding relationships of protozoa (Sandon, 1932) concerning which many facts are still obscure. It is certain, however, that protozoa are selective feeders and will either not ingest, or will excrete unchanged, certain types of bacteria.

It is tempting to suggest that a rise or fall of 500–1000 millions of bacteria may be due to a fall or rise in the number of amoebae. The food requirements of the population of amoebae in 1 gm of soil, calculated from the data of Cutler and Crump (1927) are of the order that is consistent with this view. However, marked changes in numbers of bacterial cells appeared in soil free from protozoa (and incubated at constant conditions of moisture and temperature). The possible effects of spore formation in these experiments must, however, be considered. In the experiment, *C I* and *C III*, where changes of some magnitude occurred, the inoculated organisms were either a pure culture of a spore-forming organism or a mixed culture containing a spore-forming organism. The total count represented the numbers of vegetative cells, as the spores with a subsequent disintegration of the surrounding mother-cells, would result in the substitution of unstainable spores for stainable cells, and thus be recorded as a decrease in total numbers, whereas the plate count would not necessarily be affected, since the spores would germinate in the agar media. Conversely, if sudden germination took place, an increase in total numbers would be observed, but no change would be recorded by the plate counts. It is very likely that this is what happened and that small changes in external conditions (as, for example, those caused by opening the incubator and the removal of a covering bell jar) may produce the necessary stimulus to accentuate sporulation or germination. In experiment *C IV* where a non-spore-forming, easily-stainable organism was used, no significant fluctuations on total numbers were found. However, such an alteration in the spore/vegetative-cell ratio does not explain changes in numbers of bacteria in fresh soil, whether in the plot or stored at even temperature, for in such soil the proportion of spore-forming organisms is negligible.

The present evidence suggests that an unstable equilibrium exists between the different groups comprising the micro-population of the soil. A small change in the soil environment—perhaps caused by the growth of the organisms themselves—may be sufficient to upset this equilibrium and to initiate fluctuations. Pronounced changes in moisture may produce changes in numbers clearly correlated therewith, and it seems likely that lesser changes in moisture or temperature may provide impulses capable of starting a series of fluctuations not obviously related to the primary cause.

In conclusion I should like to express my thanks to Sir John Russell for permission to work at Rothamsted Experimental Station, to Dr. H. G.

Thornton for the advice, guidance and help he has given; to Miss J. E. Rudorf and to Dr. Hugh Nicol, for their assistance during the 48-hour count, also to Mr. J. B. Marshall who made all the determinations of nitrogen of ammonia and nitrate in experiment *A V*.

TABLE X—COUNTS OF BACTERIA IN THE HALF-PLOTS FROM EXPERIMENT *A III*. SEPTEMBER 24–26, 1934

| Time | Ratio microscope counts | | | Plate counts | | |
|---|---|---|---|---|---|---|
| | Half-plot A | Half-plot B | Mean | Half-plot A | Half-plot B | Mean |
| 7 a.m. ...... | 2665·8 | 2857·0 | 2761·4 | 78·7 | 64·8 | 71·7 |
| 9 a.m. ...... | 2528·6 | 2326·2 | 2427·4 | 54·5 | 60·0 | 57·2 |
| 11 a.m. ...... | 2649·2 | 2155·9 | 2402·6 | 78·8 | 70·6 | 74·7 |
| 1 p.m. ...... | 3183·4 | 3498·6 | 3341·0 | 69·0 | 71·0 | 70·0 |
| 3 p.m. ...... | 2961·4 | 3231·0 | 3096·0 | 61·0 | 61·3 | 61·1 |
| 5 p.m. ...... | 3086·8 | 3062·4 | 3074·6 | 50·9 | 50·9 | 50·9 |
| 7 p.m. ...... | 2304·1 | 1993·7 | 2148·9 | 103·9 | 78·4 | 91·1 |
| 9 p.m. ...... | 2822·3 | 3018·5 | 2920·4 | 91·1 | 82·3 | 86·7 |
| 11 p.m. ...... | 3278·9 | 3114·6 | 3196·8 | 81·9 | 74·7 | 78·3 |
| 1 a.m. ...... | 3454·4 | 3087·7 | 3271·0 | 101·0 | 80·2 | 90·6 |
| 3 a.m. ...... | 2907·3 | 2877·2 | 2892·2 | 58·6 | 62·7 | 60·6 |
| 5 a.m. ...... | 3093·7 | 2957·2 | 3025·5 | 57·8 | 62·7 | 60·2 |
| 7 a.m. ...... | 2602·0 | 2981·5 | 2791·8 | 51·7 | 52·4 | 52·0 |
| 9 a.m. ...... | 2382·2 | 2527·6 | 2454·9 | 58·5 | 55·1 | 56·8 |
| 11 a.m. ...... | 3076·2 | 2898·1 | 2987·1 | 49·8 | 51·2 | 50·5 |
| 1 p.m. ...... | 3023·8 | 2620·4 | 2822·1 | 59·8 | 68·1 | 63·9 |
| 3 p.m. ...... | 2659·3 | 2349·7 | 2504·5 | 54·0 | 59·1 | 56·5 |
| 5 p.m. ...... | 2400·4 | 2529·9 | 2465·1 | 73·1 | 64·9 | 69·0 |
| 7 p.m. ...... | 2621·3 | 2445·0 | 2533·1 | — | — | — |
| 9 p.m. ...... | 2408·8 | 2532·0 | 2470·4 | 68·2 | 71·1 | 69·5 |
| 11 p.m. ...... | 2356·7 | 2319·5 | 2338·1 | 58·0 | 64·6 | 61·3 |
| 1 a.m. ...... | 2622·0 | 2413·4 | 2517·7 | 74·1 | 65·1 | 69·6 |
| 3 a.m. ...... | 2440·1 | 2206·6 | 2323·4 | 69·8 | 71·9 | 70·8 |
| 5 a.m. ...... | 1724·5 | 2178·9 | 1951·1 | 65·0 | 54·5 | 59·7 |

## SUMMARY

Significant changes in total bacterial numbers, as counted microscopically by the ratio method, have been shown to take place from day to day, in (*a*) soil freshly taken from the field; (*b*) soil incubated at constant temperature and moisture conditions; (*c*) sterilized and re-inoculated soil incubated at constant temperature and moisture conditions.

Using mannite-salts (referred to as " count ") and soil extract media

with the plate count method, significant day-to-day changes in bacterial numbers have been recorded in fresh soil.

Significant changes in bacterial numbers at 2-hourly intervals have been obtained in fresh soil by both total and plate count methods.

In fresh soil, fluctuations in bacterial numbers have been correlated with moisture on one occasion only, when intermittent rainfall may have been a limiting factor. In the experiment here described fluctuations were at all times independent of soil temperature changes.

By incubating soil under constant conditions of temperature and moisture it has been shown that the bacterial population may change in spite of those conditions being kept uniform.

Neither barometric pressure, light, nor time of day have had any apparent effect on the fluctuations recorded.

The numbers recorded on different plate count media from the same soil samples have usually shown no relationship to each other or to the total count; this suggests that fluctuations in total numbers are made up of a series of fluctuations occurring independently in different groups of bacteria.

In fresh soil there was, in the one test made, an inverse relationship between numbers of total bacteria and numbers of protozoa.

The evidence obtained leads to the conclusion that changes in bacterial numbers in soil are due to the existence of an innately unstable equilibrium between groups of micro-organisms which is liable to be affected by external factors such as moisture and temperature.

## REFERENCES

Allison, F. E. (1917). ' Soil Sci.,' vol. **3**, p. 37.

Brown, P. E., and Halversen, W. V. (1919). ' Iowa Agric. Expt. Stat. Bull.,' No. 56, p. 251.

Cobb, M. J. (1932). ' Soil Sci.,' vol. **33**, p. 325.

Conn, H. J. (1910). ' Zbl. Bakt.,' II, vol. **28**, p. 422.

—— (1918). ' N.Y. Geneva Agric. Expt. Sta. Tech. Bull.,' No. 64.

Conn, H. J., and Darrow, M. A. (1930). ' N.Y. Geneva Agric. Expt. Sta. Tech. Bull.,' No. 172.

Corbet, E. S. (1934). ' Soil Sci.,' vol. **38**, p. 407.

Cutler, D. W. (1920). 'J. Agric. Sci.,' vol. **10**, p. 135.

—— (1923). ' Ann. App. Biol.,' vol. **10**, p. 137.

Cutler, D. W., and Crump, L. M. (1927). ' Brit. J. Expt. Biol.,' vol. **5**, p. 155.

Cutler, D. W., Crump, L. M., and Sandon, H. (1922). ' Phil. Trans.,' B, vol. **211**, p. 317.

Cutler, D. W., and Dixon, A. (1927). ' Ann. App. Biol.,' vol. **14**, p. 247.

Eggleton, W. G. (1934). ' J. Agric. Sci.,' vol. **24**, p. 416.

Engberding, D. (1909). ' Zbl. Bakt.,' II, vol. **23**, p. 569.

Fabricius, O., and von Feilitzen, H. (1905). ' Zbl. Bakt.,' II, vol. **14**, p. 161.

Fehér, D. (1929). ' Biochem. Z.,' vol. **206**, p. 416.

—— (1930). ' Proc. Int. Cong. Forest Expt. Stat., Stockholm,' as quoted in Biol. Abs., vol. **8**, 2037.

—— (1934). ' Arch. Mikrobiol.,' 5, vol. **8**, p. 423.

Fisher, R. A. (1932). " Statistical Methods for Research Workers," Oliver and Boyd, Edinburgh.

Fisher, R. A., Thornton, H. G., and Mackenzie, W. A. (1922). ' Ann. App. Biol.,' vol. **9**, p. 325.

Germanov, F. N. (1930). ' Proc. Second. Int. Cong. Soil Sci.,' Commission III, p. 239, Moscow 1932.

Gray, P. H. H., and Thornton, H. G. (1928). ' Nature,' vol. **122**, p. 400.

Hiltner, L., and Störmer, K. (1903). ' Arb. K. GesundhtsAmt.' Biol. Abs., vol. **3**, p. 445.

Jensen, H. L. (1934). ' Proc. Linn. Soc. of N.S. Wales,' vol. **59**, p. 101.

Joffe, J. S., and Conn, H. J. (1923). ' N.Y. Geneva Agric. Expt. Sta. Tech. Bull.,' No. 97.

Kruger, W., and Heinze, B. (1907). ' Landw. Jahrb.,' vol. **36**, p. 38.

Löhnis, F. (1913) " Laboratory Methods in Agricultural Bacteriology," Griffin and Co., Ltd., London, p. 101.

Lochhead, A. G. (1924). ' Trans. Roy. Soc. Canada,' vol. **18**, p. 75.

Newton, J. D. (1930). ' Sci. Agric.,' vol. **10**, p. 361.

Paulie, E. E. (1934). ' Soil Sci.,' vol. **38**, p. 401.

Remy, T. (1902). ' Zbl. Bakt.,' II, vol. **8**, p. 761.

Richter, A. A., and Richter, B. A. (1925). ' Utchonie Zapiski. Univ. of Saratov,' vol. **4**, No. 1.

Russell, E. J., and Appleyard, A. (1917). ' J. Agric. Sci.,' vol. **8**, p. 385.

Russell, E. J., and Hutchinson, H. B. (1909). ' J. Agric. Sci.,' vol. **3**, p. 111.

—— (1913). *Ibid.*, vol. **5**, p. 152.

Sandon, H. (1932). " The Food of Protozoa," ' The Egyptian Univ.,' Cairo.

Telegdy-Kováts, L. de (1932). ' Ann. App. Biol.,' vol. **19**, p. 65.

Thatcher, L. M., and Conn, H. J. (1927). ' N.Y. Geneva Agric. Expt. Sta. Tech. Bull.,' No. 129.

Thornton, H. G. (1922). ' Ann. App. Biol.,' vol. **9**, p. 241.

Thornton, H. G., and Gangulee, N. (1926). ' Proc. Roy. Soc.,' B, vol. **99**, p. 427.

Thornton, H. G., and Gray, P. H. H. (1930). ' Proc. Roy. Soc.,' B, vol. **106**, p. 399.

—— (1934). ' Proc. Roy. Soc.,' B, vol. **115**, p. 522.

Vandecaveye, S. C., and Villanueva, B. R. (1934). ' J. Bact.,' vol. **27**, p. 257.

Waksman, S. A. (1916). ' Soil Sci.,' vol. **1**, p. 363.

Woitkiewicz, A. I. (1914). ' Zbl. Bakt.,' II, vol. **42**, p. 254.

612.015.3:595.728

# A Comparison of the Rate of Metabolic Activity in the Solitary and Migratory Phases of *Locusta migratoria*

By Colin G. Butler and Jean M. Innes

(From the Entomological Department, Zoological Laboratory, Cambridge)

(*Communicated by A. D. Imms, F.R.S.—Received November* 8, 1935)

## 1—Introduction

The occurrence of locusts in two extreme forms or phases, viz., the *phasis gregaria* and the *phasis solitaria* was first envisaged as a theoretical conception by Uvarov (1921). It was subsequently confirmed experimentally by Faure (1932), whose main results have been repeated by other observers. Given suitable temperature, humidity, and food, it is now known to be a comparatively easy matter to induce *Locusta migratoria*, for example, to develop into either phase. In a few words, individuals reared from the egg in isolation develop evident solitaria characters, whereas those reared collectively in large numbers assume the gregaria, or migratory, phase. The differences that have been described as being characteristic of these two phases are those of behaviour, coloration, and of morphological structure. It may be argued that these very evident differences are likely to be accompanied by others of a physiological nature. With the object of exploring possible physiological differences between the two phases, we have studied the rate of oxygen uptake of all the instars of *Locusta migratoria*, in both its solitary and migratory forms, using this criterion as an indication of the rate of metabolic activity.

## 2—Technique Employed

A special Barcroft apparatus was devised, with cylindrical bulbs of 5 inches in length, all the ground glass joints being sealed with mercury to prevent any leakage during the course of an experiment. The locusts used were all obtained 3 days after each had moulted and were then starved for 24 hours before the experiment. Every care was taken to use the insects in exactly the same condition, as regards feeding and development, in order to allow of a fair comparison being made.

The experimental locust was placed in a small wire gauze cage which was pressed closely around it, so as to allow only the minimum amount of movement. This cage was then suspended from a small hook in one of the vessels of the Barcroft apparatus, which contained 5 cc N/10 NaOH to absorb the carbon dioxide produced. It was found that, with this large surface of soda, no shaking was necessary to obtain complete absorption. The whole apparatus was placed in a waterbath at a temperature of 27° C, and allowed to equilibrate for 20 minutes, with the taps open, before each experiment. This temperature was chosen as being lower than that in which locusts normally live and, therefore, likely to inhibit movement to a large extent. That it was not detrimental to the locusts is shown by the fact that it was found possible to rear nymphs to the adult stage at this temperature.

The necessity for controlling the temperature accurately was emphasized by the work of Rogers (1929), who found that the rate of oxygen consumption of nymphs of the grasshopper *Melanoplus differentialis* increased with increase of temperature. The temperature of the waterbath was therefore maintained at 27° C by means of two heater bulbs controlled by an electrical thermostat and relay, adequate stirring being accomplished by a constant stream of compressed air.

The experiments were performed during August in a room specially fitted for conducting physiological experiments, facing north and so constructed that no direct light could fall upon the apparatus. The experimental locust was carefully shielded from the red glow emitted by the heater bulbs, and the room illumination was frequently checked by means of a photometer. When the illumination materially departed from the average no experiment was carried out.

After equilibration, both taps of the Barcroft apparatus were closed simultaneously and sealed with mercury to prevent leakage. The level of the fluid in the two limbs of the manometer was noted every minute for seven consecutive minutes. The mercury was then removed with a pipette and the taps opened for 1 minute, after which they were again closed and sealed. A further series of 7-minute readings was then taken with the same experimental locust. This proceeding was performed three times for each individual, and the mean oxygen uptake per minute for 7 minutes was calculated.

Ten individuals of each sex in each instar were used and the mean oxygen uptake for every batch was determined so that, altogether, thirty readings were taken in connexion with each point recorded on the accompanying graphs. For purposes of comparison of the respiratory metabolism of all instars, the results were at first expressed as oxygen uptake/unit time/gram of dry weight. This method, however, proved unsatisfactory as it appeared that the oxygen uptake for locusts of the same phase, instar, sex, and age, and under the same conditions of feeding, was higher for lighter animals and decreased progressively as the animals increased in body weight. On the other hand, individuals *having the same weight showed an identical oxygen uptake* within the limits of experimental error. It was therefore decided to make use of the formula of Meeh (1879), $\frac{(\text{Area})^3}{(\text{Weight})^2} = k$, based on the law that the surfaces of similar solids are proportional to the two-thirds power of the volume, assuming that the specific gravity is constant. The results were then expressed as oxygen uptake/unit time/unit area of the body. Bodine (1921) found that more constant values for the rate of carbon dioxide output of grasshoppers were obtained by using the two-thirds power of the weight, that is, Meeh's formula in a rough form. That the application of Meeh's formula gave much more satisfactory results in the present experiments, can be seen from Table I. To apply this formula to the results it was necessary to obtain the constant $k$, since this constant varies for each species, as shown by Simanton (1933). This was done by measuring the actual surface area of several locusts in each instar, taking their dry weights and working out the constant for each individual, the average of these values being used. It must be noted, however, that the weight of the animal in Meeh's original formula was taken as "wet weight," whereas in this work the weight was taken as "dry weight." This course may be permissible since the experimental locusts were all starved for 24 hours before experiment, and it seems likely that under these conditions the "wet weight" bears some definite relation to the

TABLE I—SHOWING COMPARATIVE RESULTS OBTAINED FROM TWO METHODS OF EXPRESSING OXYGEN CONSUMPTION IN *Locusta*

| Individual | Dry weight gm | $O_2$/gram dry weight/min cc | $O_2$/unit surface area/min cc |
|---|---|---|---|
| Migratory ♀ adult No. 3 .... | 0·276 | 0·01456 | 0·001190 |
| Migratory ♀ adult No. 7 .... | 0·607 | 0·01132 | 0·001209 |
| Solitary ♂ adult No. 1 ...... | 0·249 | 0·01325 | 0·001118 |
| Solitary ♂ adult No. 5 ...... | 0·352 | 0·01274 | 0·001108 |
| Migratory ♀ 3rd instar No. 2 | 0·040 | 0·01526 | 0·0005451 |
| Migratory ♀ 3rd instar No. 8 | 0·054 | 0·01302 | 0·0005608 |
| Solitary ♂ 1st instar No. 2 .. | 0·00026 | 0·06460 | 0·001095 |
| Solitary ♂ 1st instar No. 5 .. | 0·00028 | 0·06000 | 0·001090 |

" dry weight." Should the constant, as derived from the " dry weight," be inaccurate, the true surface area of any given locust of known " dry weight " would not be obtained. This, however, should in no wise invalidate comparisons of the oxygen uptake made between the different sexes, phases, and instars, as the percentage error in surface area due to an incorrect constant is independent of the weight and therefore affects all instars equally. The area of the body surface was determined by covering the body and amputated legs of individuals, in each instar, with the thinnest possible layer of soft wax moulded to its form, the wax then being allowed to harden slightly. The wax coat was cut with a sharp scalpel where necessary, peeled off with great care to avoid stretching, and laid out flat on paper. This was photographed and the print on standard paper was cut out and weighed. Since the scale of the photograph was not lifesize, the weight of an area of 1 sq cm (measured on graph paper) photographed on the same scale and printed on the same paper, was also obtained. By comparison of these weights the area of each wax coat (in square centimetres) was obtained. In order to check the accuracy of this method of measuring the surface area of the locust, the surface area of the same individual was measured twice in several instances and in each case the calculated error was less than 1%. It should here be mentioned, however, that the wings were amputated and not included in the surface area determinations. These organs are composed for the most part of non-cellular cuticular material only, but since blood circulates in certain of the minute channels, represented by the larger veins, a slight error is probably thus introduced.

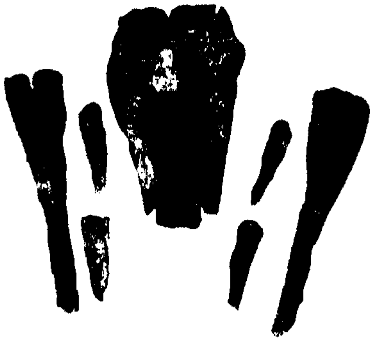


FIG. 1—Measurement of surface area of *Locusta*. Photograph of wax coatings of body and the three pairs of legs of an adult locust. The coatings were removed and flattened out.

The insects used in this work were reared from eggs laid by *Locusta migratoria* (subspecies *migratorioides*) which is kept breeding from generation to generation in the Entomological Field Station at Cambridge. The original stock consisted of eggs laid by this locust in the *phasis gregaria* near Khartoum. The supply was sent by Mr. A. H. Wood of the Gezira Agricultural Research Service to Dr. A. D. Imms who received them in June, 1933. Individuals of both phases of locusts were fed on leaves of maize until 24 hours before being required for experiment. The migratory individuals were reared in a crowded condition in large breeding cages approximately 9 cu ft in volume, each cage containing several hundred locusts. At the bottom of each cage was a metal tray containing 4 inches of damp sand in which a previous generation of locusts had deposited their eggs, and it was from these eggs that the migratory individuals were obtained. The solitary locusts were reared from the eggs of females which had been bred separately since hatching and paired with males reared in the same fashion. From the time of hatching each solitary locust was reared in a lamp-glass covered with cellophane and standing on a flowerpot full of sand, the size of lamp-glass being increased as the fifth instar was reached. Except in the first instar, when great humidity prevents successful moulting, the pots of sand were kept in a trough of water so that the humidity of the atmosphere within the lamp-glass was high. The atmosphere of the constant temperature glasshouse, in which all individuals were reared, was kept at about 60% relative humidity, and the temperature was not allowed to fall below 32° C.

It was found that the probable error of the estimations of oxygen uptake, which was determined for each individual locust separately by means of the Gaussian formula, was never more than 3%, so that our uncorrected datum can be regarded as significant.

## 3—RESULTS OF EXPERIMENTS

As will be clearly seen from the accompanying graphs, figs. 2 and 3, both male and female locusts, in the migratory phase in all instars, show a higher rate of metabolism than individuals in the same stage of growth and feeding in the solitary phase. Further, the rate of metabolism of a male locust in any instar appears to be higher than that of the corresponding female. It will be noticed that only in the first instar was there no detectable difference in the rate of metabolism between individuals in the solitary and migratory phases; but, even here, a difference between the males and females was apparent. It will further be noticed that there is a marked falling off in the rate of oxygen uptake per square centimetre of body surface from the first instar to the third instar. From the fourth instar onwards to the adult stage an increase in the rate of oxygen uptake was recorded. This decrease and subsequent increase in the rate of oxygen uptake per unit area of body surface, throughout the life cycle, has not yet been explained. Our results, however, do seem to indicate that such great morphological differences as have been described between the two phases are, in *Locusta migratoria*, probably accompanied by definite physiological changes as indicated by the difference, in the

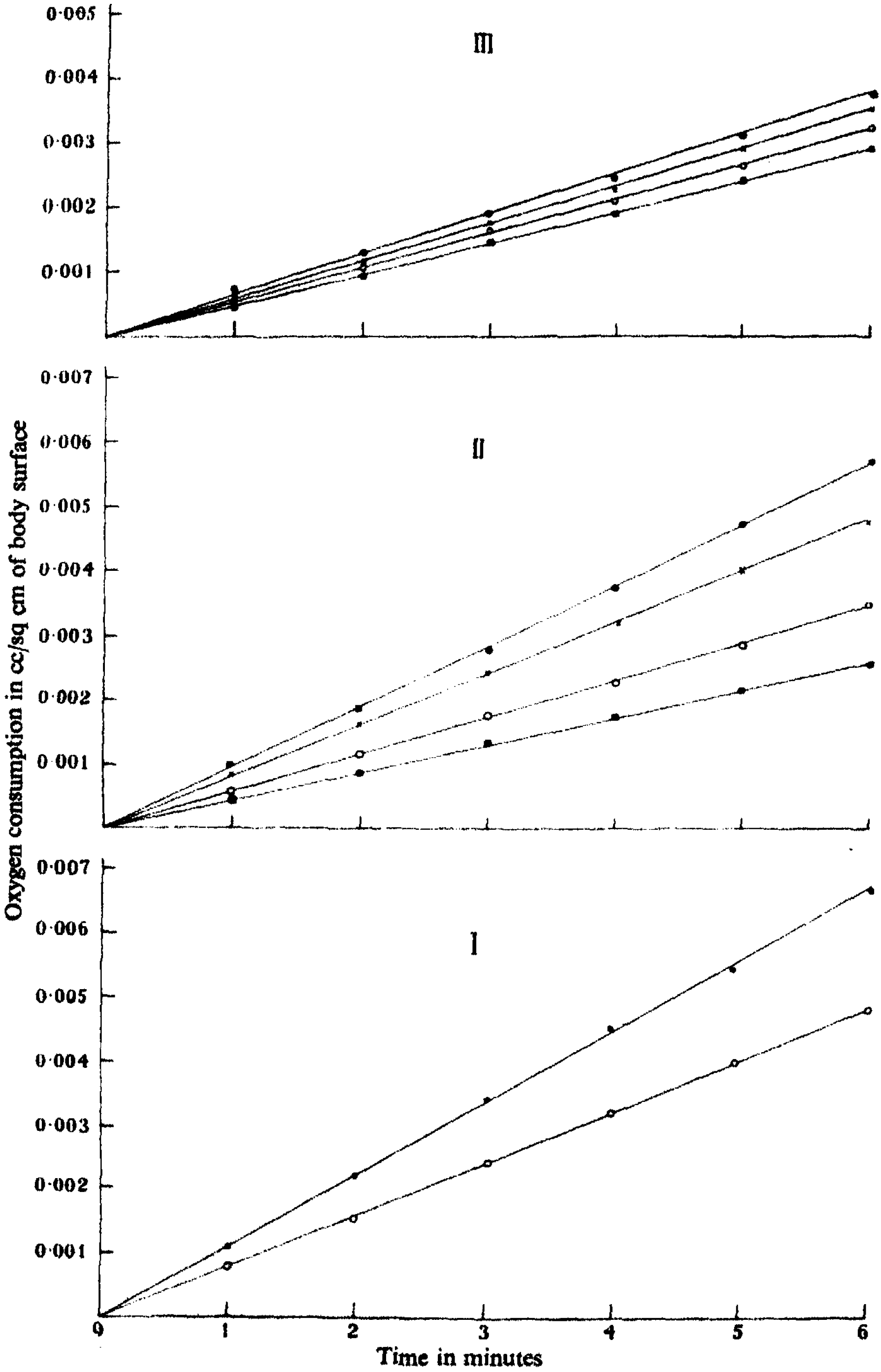


FIG. 2—Oxygen consumption in *Locusta*. I—First instar of solitary and migratory phases. As mentioned in the text, there is no significant difference in oxygen consumption in the two phases but marked sexual difference is evident in this respect. ○ = ♀; ● = ♂. II—Second instar of solitary and migratory phases. III—Third instar of solitary and migratory phases. ■ solitary ♀; ○ solitary ♂; × migratory ♀; ● migratory ♂.

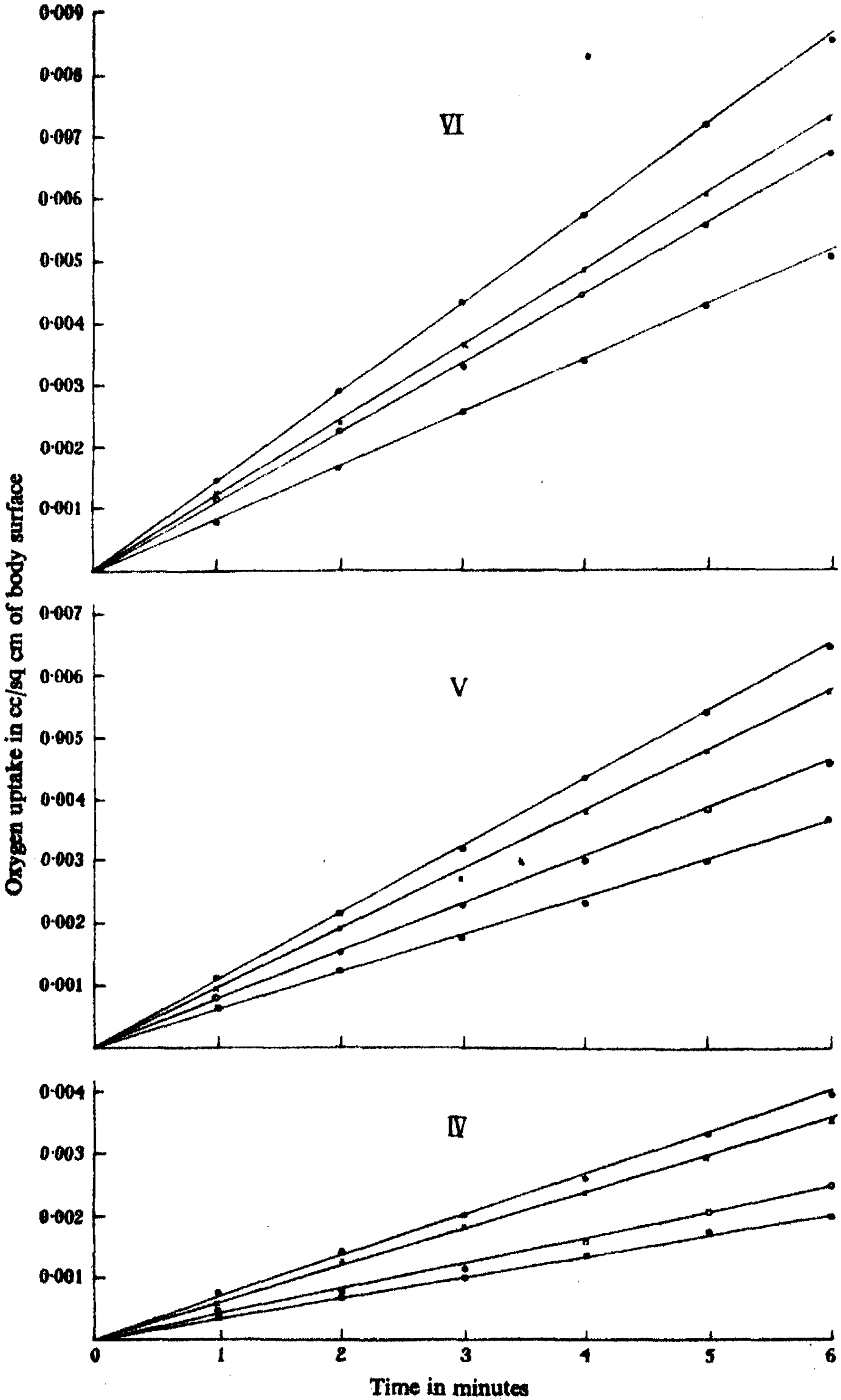


FIG. 3—Oxygen consumption in *Locusta*. IV—Fourth instar of solitary and migratory phases. V—Fifth instar of solitary and migratory phases. VI—Adults of solitary and migratory phases. ■ solitary ♀; ○ solitary ♂; × migratory ♀; ● migratory ♂.

rate of oxygen uptake per unit surface area, between solitary and migratory individuals, in all instars except the first.

## 4—DISCUSSION OF RESULTS

It is of interest to compare the above results obtained for *Locusta migratoria* with those given by other workers dealing with hemimetabolous insects. Bodenheimer (1929) working on the Desert locust, *Schistocerca gregaria*, found that young adult males had a higher rate of respiratory metabolism per unit weight than young adult females, with which our results obviously agree. Further, Bodenheimer found that from the first to fifth instars the respiratory intensity diminished progressively. From his curves it can be seen that the respiratory metabolism of the third instar, at a temperature of 29·5° C, does not fall into line with those of the first and fifth instars. A deviation is recorded which appears to be similar to that obtained for *Locusta migratoria* in the third instar. This same observer points out that the progressive diminution in respiratory metabolism through the stages, corresponds to the surface area law of Rubner (1883). This conclusion appears to be comparable with the results obtained for *Locusta migratoria* in this investigation, but is at variance with the belief expressed by Krogh (1916), on the evidence available at that time, that no reason exists for assuming a surface relation to hold for insects.

Davis and Slater (1926), working on the cockroach *Periplaneta orientalis*, found that, as the body weight increases, the rate of respiratory metabolism decreases, which is again in accordance with the results obtained with *Locusta migratoria.*

Finally Benedict and Emmes (1915) found that men have a slightly higher metabolism, per kilogram of body weight and per square centimetre of body surface, than have women of the same weight and height. Thus it appears that for these insects, although cold-blooded, the surface area law of Rubner (1883) holds, and that insect respiratory metabolism is, at least in some measure, comparable with that of vertebrates, including man.

We should like to take this opportunity of expressing our gratitude to Dr. A. D. Imms for his kind advice and supervision during the course of this work.

## 5—SUMMARY

The main object of this work is to explore possible physiological differences between the solitary and migratory phases of *Locusta migra-*

*toria* in all instars by means of accurate measurement of their oxygen consumption.

The technique employed in studying the respiratory metabolism of locusts by means of a special Barcroft apparatus is described. Two methods of rendering comparable the results of experiments on different individuals are discussed. Reasons are given for using the oxygen uptake per unit time and per unit area of body surface as the better measure of respiratory metabolism, in preference to the more usual practice of using oxygen uptake per unit time per unit gram of body weight.

The results obtained indicate that both male and female locusts, in the migratory phase, show a higher rate of metabolism than individuals in the same stages in the solitary phase. The metabolic rate in the male (in all instars) appears to be higher than that of the female. It is only in individuals in the first instar that no significant difference is revealed between those in the solitary and migratory phases: in all other instars well marked differences obtain.

It is concluded that differences in respiratory metabolism are present between the sexes and between the various instars in the migratory locust (*Locusta migratoria*). It was found that the surface area law of Rubner, which has been applied to warm-blooded animals, also holds good for *Locusta*. The extent to which these conclusions can be applied to other insects needs investigation.

## 6—LITERATURE

Benedict, F. G., and Emmes, L. E. (1915). 'J. Biol. Chem.,' vol. **20**, p. 253.
Bodenheimer, F. S. (1929). 'Z. angew. Ent.,' vol. **15**, p. 435.
Bodine, J. H. (1921). 'J. Exp. Zool.,' vol. **32**, p. 137.
Davis, J. G., and Slater, W. K. (1926). 'Biochem, J.,' vol. **20**, p. 1167.
Faure, J. C. (1932). 'Bull. Ent. Res.,' vol. **23**, pt. 3, p. 293.
Krogh, A. (1916). "The respiratory exchange of animals and man," London and New York.
Meeh, K. (1879). 'Z. Biol.,' vol. **15**, p. 425.
Rogers, E. (1929). 'Physiol. Zool.,' vol. **2**, p. 275.
Rubner, M. (1883). 'Z. Biol.,' vol. **19**, p. 536.
Simanton, W. A. (1933). 'Ann. Ent. Soc. Amer.,' vol. **26**, p. 247.
Uvarov, B. P. (1921). 'Bull. Ent. Res.,' vol. **12**, p. 135.

612.816

# Excitation and Accommodation in Nerve

By A. V. Hill, F.R.S., Foulerton Research Professor of the Royal Society

(From the Department of Physiology, University College, London)

(*Received November* 19, 1935)

## I—Introduction

When an electric current is passed through a living excitable tissue it changes the " condition " of the tissue in such a way that, if the change be in the right direction and great enough, excitation results. The " condition " is, as yet, of unknown nature: it may be an electrical potential difference: it may be an ionic concentration difference: various guesses at it have been made, but further evidence, and evidence of a more specific kind than that ordinarily considered in the theory of electric excitation, is required before a decision can be reached. The " condition," however, has many analogies with a potential in the ordinary physical sense. It will be referred to as the " local potential " V of the excitable tissue: Keith Lucas (*e.g.*, 1910) called it the " excitatory disturbance ": when we know better what it is, we can perhaps give it a better name. It will be denoted in general by V, and the resting value of V will be called $V_0$. When a current is passed into an excitable tissue V is raised at the cathode, lowered at the anode: if V is raised enough, a state of instability is reached and " excitation " occurs.

In a recent preliminary treatment of this subject (Hill, 1935, *b*) the name " cathode potential " was used. It was not realized at first that excitation at break of constant current fits into the scheme just as well as excitation at make: so that the anode must be considered equally with the cathode. " Electrode potential " has another meaning in physical chemistry and is therefore inadmissible. The changes considered occur, not in the body of the excitable cell, but around the points of entry and exit of the current. " Local potential," therefore, refers to the condition at those points.

Of the nature of the instability which occurs when V reaches a high enough value we are ignorant. There are plenty of electrical, mechanical, and chemical analogies to it, *e.g.*, in a thyratron or neon lamp flashing at a given potential difference, in a siphon emptying a tank when the water reaches a given level, in an explosion occurring at a given temperature. It is better to make no assumptions at present as to the physical nature of the happenings, until we have seen how far we can get by formal quantitative description on plausible physical lines. We shall assume that when

V reaches a certain value U " excitation " occurs, and we shall call U the " threshold." Much is known about electric excitation, and it is satisfactory to find how well this fits into a comparatively simple scheme, quantitative and physically reasonable, but with no specific physical or chemical assumptions as to the nature of the factors involved.

The " local potential " V, changed by passing a current through the excitable tissue (hereafter for brevity called " the nerve "), is known to revert to its initial value $V_0$ when the current is withdrawn. It does so gradually, not instantly. We shall assume—and the assumption will be justified by a variety of evidence later—the simplest possible law for the return of V to its original value $V_0$, viz.,

$$- dV/dt = (V - V_0)/k. \qquad (1)$$

Here $k$ has the dimensions of time; it proves to be the time-constant in excitation. The time-constant in excitation is simply that of the process by which the " local potential " tends to decay to its original value when the nerve is left to itself.*

We might have defined the time-constant in terms of the rate of change of V when a current is put into the nerve. This would have required the choice of some particular form of current. There would have been no difficulty, but it might have given the impression that $k$ has something to do with the current: it has not: it is a property of the nerve itself: it expresses the time-factor in the tendency of the nerve to revert to its resting condition.

The critical value of V required for excitation, *i.e.*, the threshold U, might have been constant and independent of the previous history of the nerve. If the current lasts only for a very short time this is true. If, however, the current lasts longer, the threshold rises, as is shown by the well-known fact that a slowly increasing current has a higher threshold than a quickly increasing one. The change of threshold is gradual, it takes place as a consequence of, and at a speed determined by, the change of " local potential " produced in the nerve by the passage of current. There is, therefore, a second time-factor in electric excitation, viz., that defining the rate of change of threshold U.

We shall use the term " accommodation " (Nernst, 1908) to describe the fact that the threshold U rises when the " local potential " V is maintained. It is known that the " accommodation " disappears of itself, *i.e.*, U reverts gradually to its original value $U_0$ when the nerve is allowed to return to its original resting state: hence we can take as the time-factor of "accommodation" that of the process by which U returns to $U_0$ when V is suddenly made $V_0$. If V is increased suddenly enough, *e.g.*, by a rapid condenser discharge, excitation will occur when $V = U_0$. Let U be the threshold at any moment in a nerve partly or fully accommodated to a current in it: then excitation will occur if $V = U$. Imagine V to be suddenly reduced to $V_0$: this could be achieved experimentally by a rapid condenser discharge in the opposite sense. Then U begins to return to $U_0$, and we shall assume that it does so in the simplest possible way, viz., exponentially with time-constant $\lambda$. Here $\lambda$ is the time-constant of decay of the "accommodation," as $k$ is that of the "local potential." We write

---

* In Keith Lucas's (1910) words " The time-factor in excitation and the summation interval are determined by a common function, namely the rate at which the excitatory disturbance subsides." Blair (1935 *a*) also remarks, " It would be much more logical to call $k$ the coefficient of subsidence of the excitatory process."

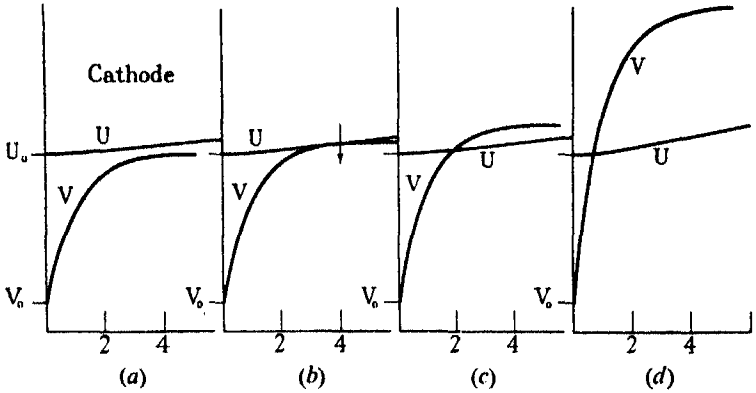
$$-dU/dt = (U - U_0)/\lambda. \tag{2}$$

This equation is for U what (1) is for V, with $\lambda$ instead of $k$. We shall see later how $\lambda$ can be measured. It is unrelated to and considerably greater than $k$, in medullated nerve 10 to 200 times as great. Had it not been, the phenomena of excitation would have been very different.

The constant $\lambda$ of "accommodation" proves to be the same as the *constante linéaire* of Fabre (1927, *a*, *b*, 1928, 1931, 1933, *a*, *b*, 1934, *d*) which he determined by the use of linear progressive currents. See § X and § XV.

When the time required for excitation ("utilization time," "*Nutzzeit*," "*durée utile*") is short enough, *e.g.*, in frog's medullated nerve at 20° C less than about 0·25 m sec, the constant $k$ only is involved: the threshold can be regarded as constant. With longer times, however, *e.g.*, with constant currents near the rheobase, with linearly or exponentially increasing currents, or with alternating current of very low frequency, $\lambda$ is involved also, and it is impossible to understand the phenomena without appreciating that *two* time-constants are required to describe them. The phenomenon of excitation at break is a necessary consequence of accommodation with a time-constant ($\lambda$) much greater than that ($k$) of excitation proper. If $\lambda$ were always a constant multiple of $k$ the two time-factors might effectively be treated as one. We shall see, however, that $\lambda$ can vary over a wide range independently of $k$, *e.g.*, as the result of changing the Ca ion concentration in the tissue fluid. Hence $\lambda$ must be regarded as an independent constant.

In figs. 1 to 4 the process of excitation by constant current is illustrated. The case taken is that of a frog's sciatic nerve at 4° C for which $k = 1$ m sec. In figs. 1 and 2 the value of $\lambda$ is an ordinary one for this temperature, about 50 m sec. In fig. 1 excitation at "make" is shown; in fig. 2, excitation at "break." The problem is, will V catch U, or will U have time to escape? V and U are calculated from equations (12) and (13) below. V starting from $V_0$ rises exponentially to a value depending on the current. U starting from $U_0$ rises along a more complicated curve, of which only a part can be shown, to a final value depending on the final steady value of V. In fig. 1*a*, the current is supposed adjusted to such a value (= 1) that, in the absence of accommodation, threshold excitation would result. V actually reaches $U_0$, but by then the threshold has risen sufficiently to prevent excitation. To avoid this effect the current has to be 8% greater, and in fig. 1*b* the threshold is just reached in 4 m sec: which—to use Gildemeister's term (1913)—is the *Hauptnutzzeit*. In fig. 1*c* a somewhat stronger current (1·2) is used, and V crosses the threshold in 1·85 m sec. In fig. 1*d* a much stronger current (2·0) is used, and V crosses the threshold in 0·7 m sec. The stronger the current, the shorter will be the utilization time. The strength-duration curve, for constant current pulses, merely gives the time, for any given current, at which V crosses U.



(*a*) (*b*) (*c*) (*d*)
Milliseconds

(*a*) current = 1, (*b*) current = 1·08, (*c*) current = 1·2, (*d*) current = 2·0

FIG. 1—Rise of "local potential" V and of "threshold" U, at the cathode, for constant current suddenly applied. Calculated for frog's nerve at 4° C, ordinary "accommodation," $k = 1$, $\lambda = 50$ m sec: various currents, as shown. Current 1 would be "rheobase" apart from accommodation. The actual rheobase is 1·08, and its "utilization time" is shown by an arrow.

In fig. 1 the curve for U is not continued beyond 6 m sec: with $\lambda = 50$ m sec it would take 230 m sec to be within 1% of its final value. A more complete curve is shown in fig. 4, for which $\lambda$ is smaller.

The process shown in fig. 1 represents what is supposed to take place at the cathode, where V rises towards U, initially much faster than U, and may catch U if the current used is strong enough. It is almost certain that similar processes will take place, in the opposite sense, when the current is reversed, and in fig. 2 (left) we have the case of the anode, at which V and U both fall, instead of rise, when the current is made. In this case, however, there is no chance of V catching U, since the more speedy of the two has the start. There can never be excitation at the anode at make.

After an appropriate time, in this case about 0·23 sec, the system has settled down to its new condition: " accommodation " is complete: the " local potential " has long been steady: V and U on the right of fig. 2 are shown by the broken lines. Let the current now be broken: V and U immediately begin to return to their initial values. V gets within 1% of $V_0$ in 4·6 m sec; U takes 230 m sec to arrive within the same distance of $U_0$. The much greater speed of V allows it to cross U and excitation occurs at 0·7 m sec. Thus excitation at break must occur with currents sufficiently strong to bring U well down into the region which V must traverse in returning to $V_0$.

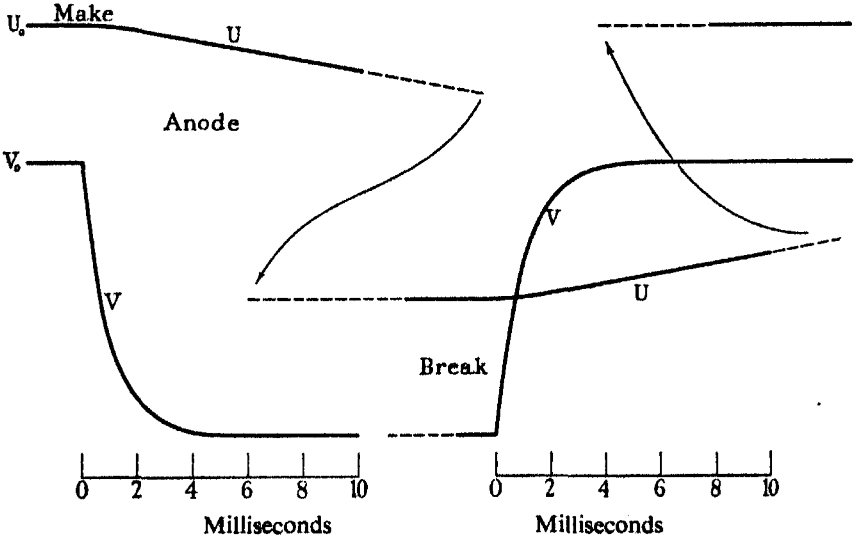
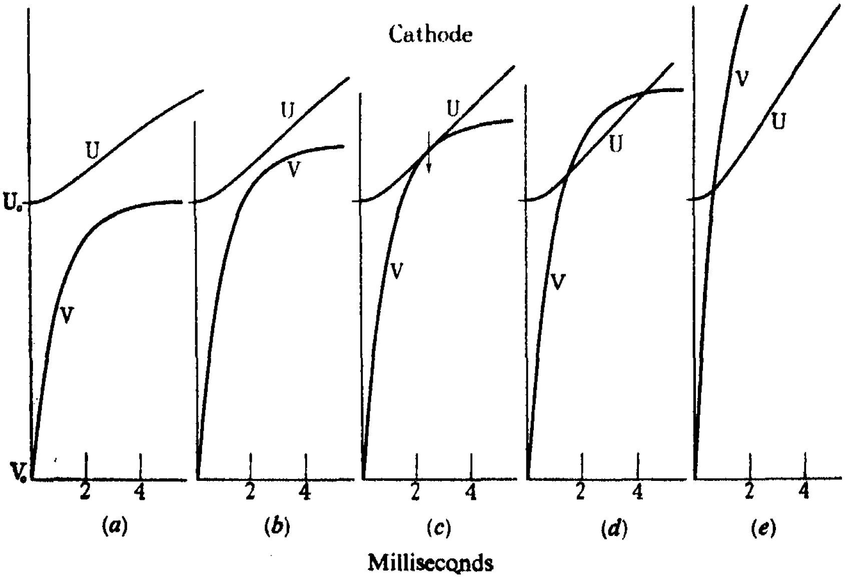


FIG. 2—Fall of " local potential " V and of " threshold " U, at the anode, for constant current suddenly applied. Ordinary accommodation: conditions as in fig. 1. No excitation at make. After accommodation is complete, current suddenly broken, and the nerve returns to its initial condition. V crosses U and excitation at break results.

In fig. 3 another case of excitation at make is shown, this time with very rapid accommodation. Here as before $k = 1$ m sec, but $\lambda = 10$ m sec instead of 50. The current (= 1) which would be the rheobase apart from " accommodation " is shown, fig. 3*a*, never to be near the threshold. Even a current 20% stronger (= 1·20) still fails, fig. 3*b*. The threshold current is 1·29, and the *Hauptnutzzeit* = 2·6 m sec, fig. 3*c*. In fig. 3*d* a current of 1·4 excites in 1·5 m sec: in fig. 3*e* a current of 2·0 excites in 0·7 m sec. The phenomena are just the same as in fig. 1, but the figure is drawn on a different scale, and " accommodation " occurs more quickly.



(*a*) current = 1·0, (*b*) current = 1·2, (*c*) current = 1·29, (*d*) current = 1·40,
(*e*) current = 2·0

FIG. 3—Rise of " local potential " V and of " threshold " U, at the cathode, for constant current suddenly applied. Calculated for frog's nerve at 4° C, very rapid " accommodation," $k = 1$, $\lambda = 10$ m sec. Current 1 would be " rheobase " apart from accommodation. The actual rheobase is 1·29, and its utilization time is shown by an arrow.

In fig. 4, for the same case, are shown, as in fig. 2, the changes of V and U at the anode. Again it is obvious that V cannot be caught by U, the quicker by the slower. Excitation does not occur, a steady state is reached in respect of V in 5 m sec, in respect of U in 50 m sec. When this is reached the current is broken. The curves to the right show what happens. V crosses U in about 1·3 m sec, excitation occurs at break.

It is unnecessary, therefore, to provide any special explanation of excitation at break. If we are right in thinking of " local potential " and " threshold " varying, under the influence of current traversing the tissue, in the way described, and if we admit—as on almost any physical theory we are bound to do—that changes in either must be similar but opposite in sign, when the current is reversed, then excitation at break is an inevitable consequence of " accommodation ": only in a tissue which did not " accommodate " at all would it not happen. Moreover, for excitation at break (not at subsequent make: Lucas 1907, *a*) the strength-duration relation should be the same, with the same time-constant, as for excitation at make.

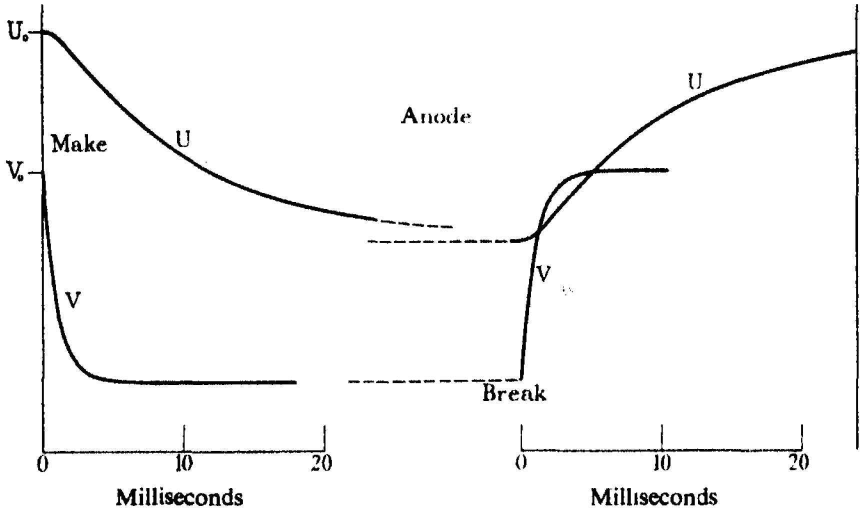
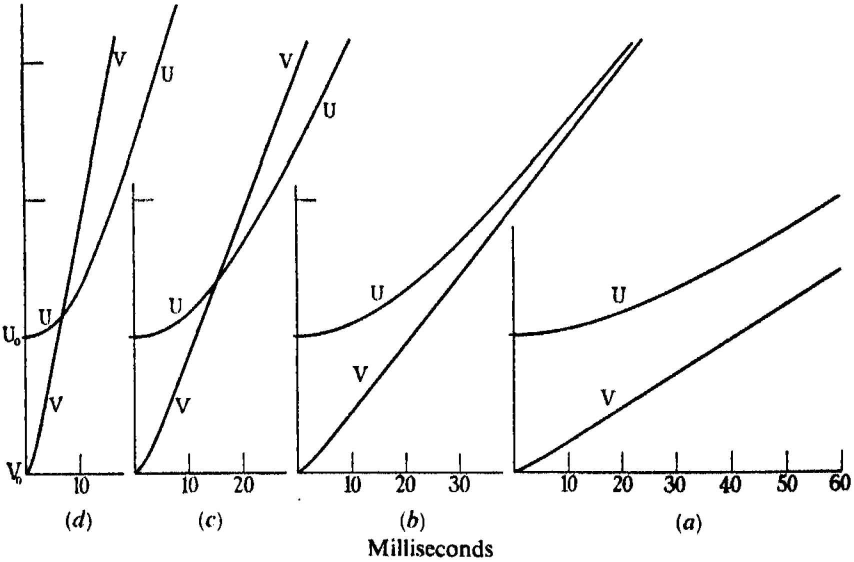


Fig. 4—Fall of " local potential " V and of " threshold " U, at the anode, for constant current suddenly applied. Very rapid accommodation, conditions as in fig. 3. No excitation at make. After accommodation is complete, current suddenly broken, and the nerve returns to its resting condition. V crosses U and excitation at break results.

The phenomena of " accommodation " in nerve, although occurring with constant currents, and although the basis of excitation at break, are best known and most strikingly demonstrated with slowly increasing currents. It has been known for many years that if a current is to excite it must increase at more than a certain rate: that the more slowly it rises the stronger it must be to attain the threshold. Von Kries (1884), Lucas (1907, *b*), Fabre (1927 to 1934), and Blair (1935, *b*), used linearly increasing currents, and Lucas showed that a " minimal gradient " is necessary, below which, however strong it may ultimately become, a current cannot excite. Gildemeister (1904, 1913), Lapicque (1908, *a*, 1926), Cardot and Laugier (1913), Delville (1934), Schriever (1930, 1932, *a*), and others used exponentially increasing currents, and studied the relation between threshold strength and rate of rise, utilization time, etc. Other forms of current rise may be used, but these are typical and easy to treat theoretically: the results of all are in principle the same.

In fig. 5 the case of " linear progressive currents " is illustrated graphically, for various gradients. The constants taken are for rather rapid " accommodation " in a frog's nerve at 4° C, $k = 1$ m sec, $\lambda = 20$ m sec. With these currents the minimal current gradient can be shown (equation (24) below) to be 50; in other words, such a current would reach 50 times the rheobase in 1 sec. The changes of " local potential " V and " threshold " U are shown in fig. 5 for 4 current gradients, 25, 50, 100, and 200 respectively. The curves are calculated from equations (22) and (23) below.



(*d*) gradient = 200, (*c*) gradient = 100, (*b*) gradient = 50, (*a*) gradient = 25

FIG. 5—Rise of " local potential " V and of " threshold " U, at the cathode, for linear progressive currents of various gradients. Calculated for frog's nerve at 4° C, rather rapid " accommodation," $k = 1$, $\lambda = 20$ m sec. The " gradient " is the number of times the rheobase which the current would attain in 1 second if it continued. The " minimal gradient " for excitation is 50.

The current of gradient 25, fig. 5*a*, does not excite at all: V starting behind U never succeeds in catching it. The current of gradient 50, fig. 5*b*, just excites after a rather long (mathematically infinite) time. The current of gradient 100, fig. 5*c*, excites at 15 m sec, V catching U before the latter has time to get very far. The current of gradient 200, fig. 5*d*, excites in 7 m sec, V catching U still earlier. The relation between the final strength of a linear progressive current at which excitation is achieved, and the time taken (the former is the product of the gradient and the time) can be separately calculated, or observed experimentally, *see* fig. 9, but it could, in principle, be deduced graphically from the system of curves in fig. 5.

In fig. 6 the case of exponentially increasing currents is illustrated (*see* equations (25) and (26)). The constants taken are the same as in figs. 1 and 2, for an average frog's nerve at 4° C, with $k = 1$ m sec, $\lambda = 50$ m sec. The current is supposed to rise exponentially with a time-constant of 100 m sec, or expressing $t$ in m sec, as

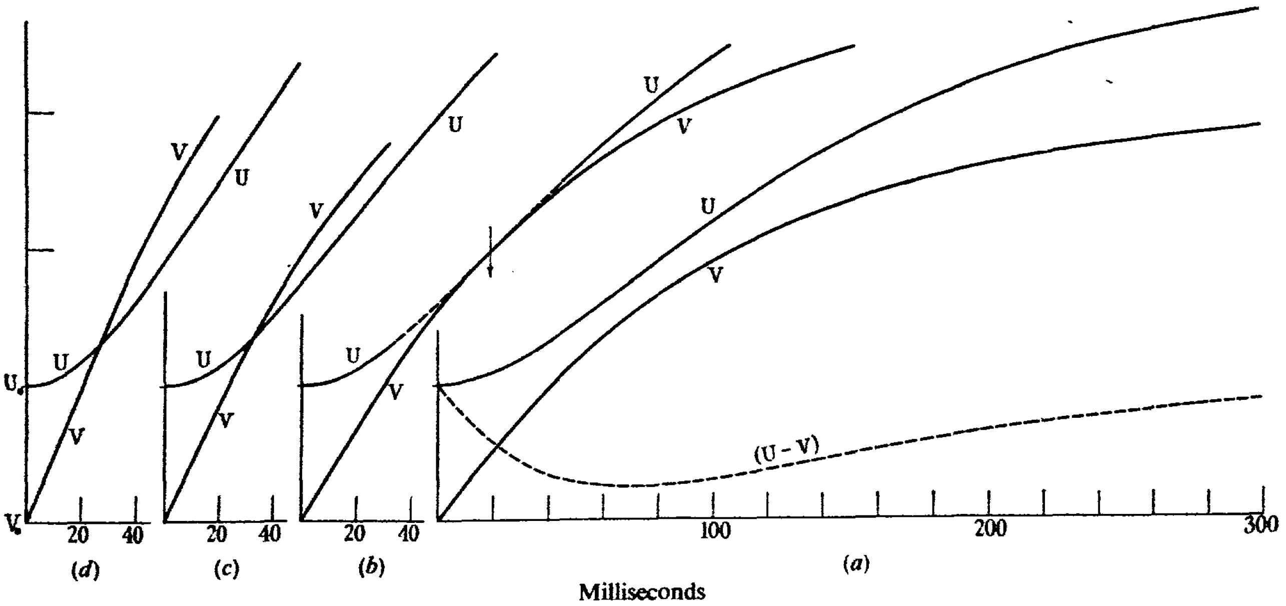
$$I = I_2 (1 - e^{-t/100}),$$

where $I_2$ is its final value. The relative values of $I_2$ chosen are 3, 4, 5, and 6 respectively. Apart from a short lag (invisible in the curves) at the start, the " local potential " V rises nearly exponentially to its final value, which it reaches within 1% (for this particular case) in 460 m sec. The " threshold " U follows an S-shaped curve, starting off slowly, reaching its greatest speed at about 70 m sec, then gradually passing to its fully accommodated level.

For final current strength $I_2 = 3$, fig. 6*a*, excitation never occurs, V and U coming nearest to one another at about 70 m sec, U then getting away. For $I_2 = 4$, fig. 6*b*, excitation just occurs at about 70 m sec, the " utilization time " for a threshold current of this form (*see* equation (27) below): the curves for V and U just touch, and then U draws away again, as in fig. 1*b*. For $I_2 = 5$ excitation occurs earlier: V crosses U at about 33 m sec. For $I_2 = 6$ excitation is earlier still, V crossing U at about 27 m sec. The stronger the current, the shorter is the utilization time.

A similar family of curves could be drawn for any speed of exponential current rise. The shorter the time-constant of the rise, the earlier will excitation occur, and the less will be the strength of current required. On the other hand, if " accommodation " is quicker, *i.e.*, $\lambda$ less, the curve for U will rise more rapidly and the strength of current required be greater.

It will be shown below, and it is almost obvious intuitively, that a very rapid pulse, or condenser discharge, or induction shock, applied at any moment, will instantly raise the value of V without immediately disturbing U. In fig. 7*a*, the case is illustrated of a frog's nerve at about 15° C with very quick " accommodation," $k = 0 \cdot 5$, $\lambda = 5$ m sec, to which a very rapid shock, 90% of threshold, is applied at time zero (*see* equations (3) and (10) below). The V-curve is instantly raised to within



(*d*) $I_2 = 6$, (*c*) $I_2 = 5$, (*b*) $I_2 = 4$, (*a*) $I_2$ = final count = 3

FIG. 6—Rise of " local potential " V and of " threshold " U, at the cathode, for exponentially progressive currents, all of the same time-constant of rise (100 m sec), but of different strengths. Calculated for frog's nerve at 4° C, ordinary " accommodation," $k = 1$, $\lambda = 50$ m sec. Strength 3 does not excite: strength 4 just excites at the utilization time shown by the arrow. The broken line in (*a*) is for (U − V), and represents (reciprocally) the changes of excitability during the flow of the current.

10% of $U_0$, and then falls exponentially with time constant *k*. The fact that V is above its resting level for several milliseconds causes the threshold U to rise, as shown in the figure, and only after 15 to 20 m sec (with these constants) will U have returned to $U_0$. This, however, does not affect the fact that a very rapid discharge instantly raises V without immediately affecting U; it only emphasizes the condition that the discharge must be rapid.

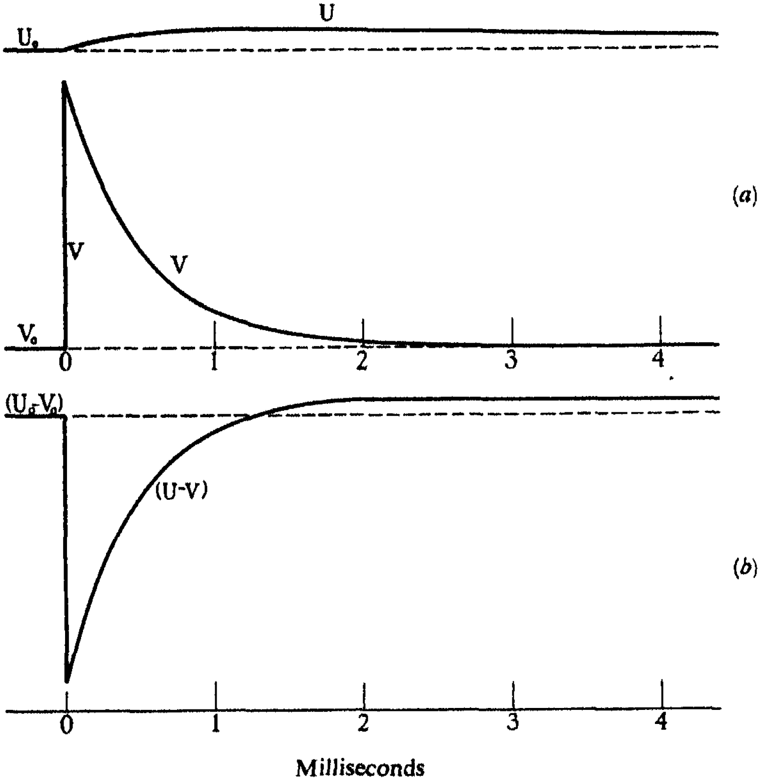


FIG. 7—(*a*) Rise of " local potential " V and of " threshold " U, at the cathode, for an " instantaneous " pulse of current of 90% threshold strength. Calculated for frog's nerve at 15° C, very rapid " accommodation," $k = 0 \cdot 5$, $\lambda = 5$ m sec. V rises instantly to within 10% of $U_0$ and then falls exponentially with time-constant *k*. (*b*) (U − V) for the same, showing that the " excitability " is instantly raised by a sub-threshold shock: then falls, to reach its original value in 1·3 m sec: then passes through a long phase of slight " post-cathodal depression " (Erlanger and Blair).

It is possible therefore by simple means to find the distance between the curves of U and V at any moment: (U — V) is directly proportional to Q, the quantity of electricity " instantaneously " discharged which can just effect excitation. By suitable arrangements the " excitability " of the nerve can thus be tested experimentally at various moments, as was done by Gildemeister (1908), Bishop (1928), Erlanger and Blair (1931, *a*, *b*), Rushton (1932), Fabre (1934, *b*, *c*). In fig. 6*a*, the broken line represents (U – V) for an exponentially increasing current, and this is the type of curve which is obtained by actual experiment. An analogous curve for (U – V) might be drawn, from fig. 5, for linear progressive currents, for comparison with Fabre's results: or, for constant currents, from figs. 1 to 4, for comparison with those of Rushton (1932) and of Erlanger and Blair (1931, *b*). The case of short pulses of current is illustrated in fig. 7*b*, where (U – V) follows a characteristic course, which explains some of the results of Gildemeister (1908) and of Erlanger and Blair (1931, *a*) on sub-threshold " excitation " by induction shocks or condenser discharges. Although, therefore, we cannot observe U and V directly, we can measure the difference between them at any moment, and compare the result with experiment. The comparison is a satisfactory test of the theory.

The ordinary strength-duration curve deals with short times and refers chiefly to the changes of " local potential " V under the influence of applied current: the threshold U is assumed to be constant. The time-constant measured from the ordinary strength-duration curve is $k$. It is possible, however, to make a strength-duration curve which depends upon U and $\lambda$, not upon V and $k$. For example, Lucas (1907, *a*) investigated the strength-duration relation for a gap in a current passing through a nerve, excitation being, not at break but at subsequent make. The time-constant of this relation is $\lambda$, that of " accommodation." The converse experiment, that in which excitation at break is examined after a pulse of constant current of variable strength and duration, was made by Cardot and Laugier (1912, *a*, *b*), and also gives a relation which depends only upon the time-constant $\lambda$. The fact that these two types of strength-duration relation, involving respectively $k$ and $\lambda$, are similar in general form though different in time-scale, is due, not to any relation between $k$ and $\lambda$ which are independent, not to two different "excitabilities " with different time-constants, but simply to the fact that V and U both tend to revert exponentially to their original values when the constraint displacing them is removed.

## II—THE DEVELOPMENT OF " LOCAL POTENTIAL " V UNDER THE INFLUENCE OF A CURRENT

The fact that V tends to decay of itself (equation (1)) affects the development of V under the influence of a current. In one case only (*see* fig. 7) does the magnitude of V attained depend only upon the applied current, and not at all upon the constant $k$ of decay: that is, when the current is applied for a very short time, *i.e.*, short compared with $k$. A quantity Q of electricity discharged through a nerve in a very short time causes an instantaneous rise $\Delta V$ in the "local potential," which then proceeds to decay with time-constant $k$.

Much recent evidence has established the fact that with discharges of very short duration a given *quantity* of electricity (and not for example a given *energy*) is required for excitation. Fabre and Swyngedauw (1933) and Fabre, Quesnoy and Berteaux (1934) proved this for frog's nerve, both for condenser discharges and for rectangular pulses: and showed that the *same quantity* is required for both forms of stimulus. Scott (1934), Bugnard and Hill (1935, *b*), and Rosenberg (1935) examined the matter more closely with condenser discharges, and found that the strength-duration (voltage-capacity) curve always converged to a line of constant quantity at short times. They worked with various nerves, from cat, dog, frog, cuttle-fish, and spider-crab. The constant-quantity relation for short times is very accurately obeyed, and it forms a crucial test for any excitation formula.*

For very *short* times the *quantity* Q of electricity required for excitation is constant: for very *long* times the *current* $I_0$ required for excitation is constant; it will be shown below that, neglecting accommodation, $Q/I_0 = k$, the time-constant of excitation.

We have assumed that, with pulses of very short duration, excitation occurs when the rise $\Delta V$ of "local potential" reaches a certain value. We know experimentally that under the same conditions excitation occurs when the quantity Q of electricity reaches a certain value. We are justified therefore in putting $\Delta V$ proportional to Q, or since $\Delta V$ decays with time-constant $k$, we may write, for instantaneous discharge,

$$\Delta V = bQe^{-t/k}, \tag{3}$$

where $b$ is a constant. Equation (3) represents the fundamental property of the local potential: it is raised instantly and proportionally by an instantaneous discharge through the nerve: it then decays with time-contant $k$.

---

* Colle (1933, *a*, *b*) has examined the voltage-duration relation for the threshold excitation of heart muscle. The log (volts) − log (duration) curve was very similar to that for nerve, but the final slope, instead of being unity, was about 0·7. This does not correspond to a constant quantity, nor to a constant energy, in the discharge. The only resistance in the circuit was that of the tissue itself, which would probably be less for short times and high volts. It is possible that if a large resistance had been introduced, or current measured instead of voltage, the usual relation of constant quantity at short times would have been found. Blair's (1932, *a*, 1934) equation for the strength-duration curve by constant current pulses does not survive the crucial test of giving the constant quantity relation at short times: nor does Lapicque's (1926, p. 214) "canonical curve."

From equation (3) we can deduce the " local potential " V at any time for a current I of any form. Put $Q = I_\theta \delta\theta$, where the suffix $\theta$ denotes that I is a function of $\theta$, the time. Then equation (3) becomes

$$\delta V = bI_\theta \delta\theta e^{-(t-\theta)/k}. \tag{3'}$$

This holds from time $t = \theta$ onwards. By integration we obtain

$$V - V_0 = be^{-t/k} \int_{\theta=0}^{\theta=t} I_\theta e^{\theta/k} d\theta. \tag{4}$$

This equation can generally be integrated, and so the " local potential " calculated for any form of current. If the times involved are short enough, so that accommodation is negligible, excitation occurs when $V = U_0$. If the times are longer, then the threshold rises, and excitation results only if V becomes equal to U, the new and higher threshold of figs. 1 to 7.

## III—The Rise of the Threshold

The change of threshold (" accommodation ") might be due directly to the current itself, if so U and I would be related in just the same kind of way as V and I, but with $\lambda$ instead of $k$. Equation (4) for V would become for U

$$U = U_0 + b'e^{-t/\lambda} \int_{\theta=0}^{\theta=t} Ie^{\theta/\lambda} d\theta.$$

A scheme could be worked out on these lines.

" Accommodation," however, is probably of the same general nature as " excitation " itself, only occurring slowly and continuously, instead of explosively. Excitation is caused by a sufficient rise in the " local potential " V: it is natural, therefore, to suppose that " accommodation " is caused, not directly by I, but also by the rise of V. In a mixture of hydrogen and oxygen (Hill, 1910, p. 208) an explosion occurs if the temperature is suddenly raised to more than a critical value: if, however, the temperature is raised slowly the gases combine, without explosion, and the " threshold " gradually rises as the gas mixture becomes weaker. On this view the rise of U, which we call " accommodation ", is to be ascribed, not to I the current as such, but to $(V - V_0)$ the rise of " local potential " result from it.

Let us assume that the rate of change of " threshold " at any moment, viz., $dU/dt$, depends upon two factors, (i) a tendency to rise at a rate proportional to the value of $(V - V_0)$ at that moment, and (ii) a tendency to decay of itself to its original value $U_0$, in an exponential manner with time-constant $\lambda$. We write,

$$\frac{dU}{dt} = \frac{V - V_0}{\beta} - \frac{U - U_0}{\lambda}, \tag{5}$$

where $\beta$ is a constant of the dimensions of time. The solution of this differential equation is

$$U - U_0 = \frac{e^{-t/\lambda}}{\beta} \int_{\theta=0}^{\theta=t} (V_\theta - V_0)\, e^{\theta/\lambda}\, d\theta, \tag{6}$$

where the suffix $\theta$ denotes that V is a function of $\theta$. This equation, like (4) above, can generally be integrated.

In dealing with " accommodation " we shall adopt a simplified scheme, one in which the well-known electrotonic changes of excitability associated with the name of Pflüger do not occur. According to Eichler (1933) at a low temperature (about 5° C) the usual electrotonic rise of excitability at the cathode, during the passage of a constant current, and the electrotonic fall of excitability at the anode, are absent. Eichler's results have been confirmed by Solandt (1936). According to Chweitzer (1935) and Bouman (1936) an increase in the K-ion concentration around a nerve may abolish or reverse the ordinary electrotonic changes of excitability. It is probable, therefore, that these changes are of a secondary nature, for the ordinary phenomena of excitation and accommodation can occur in their absence. We shall assume, therefore, for simplicity, that in a nerve " fully accommodated " to the passage of a constant current the excitability is the same as it was originally, *i.e.*, that the *extra* current required to excite is the same as the original rheobase. If, therefore, $U_1$ and $V_1$ are the final values of U and V, after full " accommodation " has been reached, we must have $U_1 - V_1 = U_0 - V_0$. This leads to a simplification of equation (6) by the elimination of $\beta$, and makes the mathematics more manageable.

If a constant current be applied to a nerve for a sufficient time, the " threshold " U alters, owing to " accommodation," and its final value is obtained by integrating equation (6) and then putting $t = \infty$. We obtain $U_1 - U_0 = \lambda/\beta\,(V_1 - V_0)$. We shall assume, however, a state of " normal " accommodation, in which $U_1 - V_1 = U_0 - V_0$. This requires that $\lambda/\beta = 1$, or $\beta = \lambda$. Equation (6) then becomes,

$$U - U_0 = \frac{e^{-t/\lambda}}{\lambda} \int_{\theta=0}^{\theta=t} (V_\theta - V_0)\, e^{\theta/\lambda}\, d\theta. \tag{7}$$

Since we can calculate $(V - V_0)$ from equation (4) for any current-form I, we can, by *inserting it in equation* (7) *obtain the value of* $(U - U_0)$ as a function of the time. This provides a general solution of the problem of excitation.

### IV—The General Problem of Excitation by a Current of Any Form

V and U can be calculated from equations (4) and (7) for a current I of any form. For excitation V must become equal to or greater than U. For critical or threshold excitation, however, not only must $V = U$ but $dV/dt = dU/dt$. The V curve in fact must creep up to the U curve and touch it, as in figs. 1, 3, 5, and 6; if it crosses it, the stimulus is above threshold and unnecessarily strong. The condition $V = U$ leads to the equation

$$V_0 + be^{-t/k} \int_{\theta=0}^{\theta=t} I_\theta \, e^{\theta/k} \, d\theta = U_0 + \frac{e^{-t/\lambda}}{\lambda} \int_{\theta=0}^{\theta=t} (V_\theta - V_0) \, e^{\theta/\lambda} \, d\theta, \qquad (8)$$

where V is calculated from equation (4). The condition $dV/dt = dU/dt$ leads, on differentiating equation (8), to the result,

$$-(V - V_0)/k + bI = -(U - U_0)/\lambda + (V - V_0)/\lambda.$$

Since $V = U$ in this case we finally obtain

$$(V - V_0)/k = bI - (U_0 - V_0)/\lambda. \qquad (9)$$

Equations (8) and (9) are the general conditions for threshold excitation. We shall now consider some particular cases.

### V—A Pulse of Very Short Duration

In this case, from equation (3)

$$V - V_0 = bQe^{-t/k}$$

and excitation occurs immediately if $V = U_0$. From equation (7) we then obtain, on integrating,

$$U = U_0 + \frac{bQ}{\lambda/k - 1}\left(e^{-t/\lambda} - e^{-t/k}\right) \qquad (10)$$

and by subtraction,

$$U - V = U_0 - V_0 + bQ\left(\frac{e^{-t/\lambda}}{\lambda/k - 1} - \frac{e^{-t/k}}{1 - k/\lambda}\right). \qquad (11)$$

These equations were used in constructing fig. 7. In that case Q was taken as the pulse which was 90% of threshold, and $bQ$ was put equal to $0{\cdot}9\,(U_0 - V_0)$. The changes of excitability following such a pulse have been discussed above.

In fig. 7, *b*, we see that a second very rapid discharge will " sum " with the first, provided that it comes while $(U - V)$ is less than its original value. Lucas (1910) examined the summation interval in muscle and nerve, and records the very pertinent fact that, in both, treatment with an excess of Ca-ions largely reduces the interval. Lucas (1908 *b*) also showed that Ca greatly increases the minimal current gradient of muscle. Kahn (1911) obtained a similar result on muscle with exponential progressive currents. Solandt in the following paper will show that in nerve Ca has a large effect in increasing the speed of accommodation. Now a decrease in $\lambda$ would produce, in fig. 7, a more rapid and extensive increase in U so that $(U - V)$ would more quickly return to and cross its original level. Increased speed of " accommodation," therefore, is bound to cause decreased " summation interval." All these effects of Ca are interrelated.

## VI—A CONSTANT CURRENT OF INDEFINITE DURATION

Here equation (4) gives

$$V = V_0 + bkI\,(1 - e^{-t/k}) \tag{12}$$

and equation (7),

$$U = U_0 + bkI\left[1 + \frac{e^{-t/k}}{\lambda/k - 1} - \frac{e^{-t/\lambda}}{1 - k/\lambda}\right]. \tag{13}$$

These equations were used in the construction of figs. 1 to 4. In the absence of " accommodation " excitation occurs when $V = U_0$, so that the " true rheobase " $I_0$ is given by putting $V = U_0$ and $t = \infty$ in equation (12); hence $U_0 - V_0 = bkI_0$. Taking account of " accommodation," threshold excitation requires that $V = U$ and $dV/dt = dU/dt$. Applying these conditions to equations (12) and (13) we find that the ratio of the " observed rheobase " $I_1$ to the " true rheobase " $I_0$ is given by

$$I_1/I_0 = (\lambda/k)^{\frac{1}{\lambda/k - 1}} \tag{14}$$

and that the time $t_1$ at which rheobasic excitation takes place (Gildemeister's (1913) *Hauptnutzzeit*) is,

$$t_1 = \frac{\log_e \lambda/k}{1/k - 1/\lambda}. \tag{15}$$

It will be shown in the following paper by Solandt, for frog's medullated nerve, that a usual value of $\lambda/k$ is about 40–80, but that variations from 10 to 200 are possible. Table I gives calculated values of $I_1/I_0$ and of $t_1/k$, for various values of $\lambda/k$, and also values of $t_1$ for $k = 0{\cdot}40$ m sec, which corresponds to a frog's nerve at about 20° C (" chronaxie " = 0·28 m sec).

TABLE I

| $\lambda/k$ ...... | 10 | 15 | 25 | 40 | 60 | 100 | 150 | 200 |
|---|---|---|---|---|---|---|---|---|
| $I_1/I_0$ ...... | 1·29 | 1·21 | 1·14 | 1·10 | 1·07 | 1·05 | 1·035 | 1·03 |
| $t_1/k$ ...... | 2·56 | 2·91 | 3·35 | 3·79 | 4·17 | 4·66 | 5·04 | 5·33 |
| $t_1$ (m sec) .. | 1·02 | 1·16 | 1·34 | 1·52 | 1·67 | 1·86 | 2·04 | 2·13 |

Even with very slow accommodation (large $\lambda/k$) the difference between the " observed " and the " true " rheobase is not quite negligible, while it becomes considerable when the accommodation is rapid. It is noticeable that the " *Hauptnutzzeit*," $t_1$, can vary in the ratio only of 2 : 1 for extreme variation of $\lambda$. The absolute values of $t_1$, calculated for the typical case of a frog's nerve at 20° C, are slightly less than those commonly recorded. With an ordinary speed of " accommodation " the calculated value, for the case considered of a frog's nerve at 20° C, is about 1·6–1·9 m sec. According to Fick (1864) the maximum effect of a constant current is reached in 2 m sec. Lapicque (1908 *b*, p. 614) records that in a frog's sciatic at 12·5° C prolonging the current beyond 3 m sec has no further effect. Gildemeister (1913, p. 369) gives 2–3 m sec as the limit; Bishop (1928, p. 422) the same. The agreement, however, is reasonably good: as Lapicque remarks (Monnier, 1934, *a*, p. xii) " *une formule approchée et maniable nous suffit, pourvu qu'elle traduise avec une approximation suffisante le propriété physiologique que nous voulons considérer.*"

Mines (1908, p. 439) showed that in frog's muscle the limiting duration of a threshold constant current (the *Hauptnutzzeit*) is much less after treatment with an excess of calcium. Gildemeister (1913) refers to the same fact. This is another example of the effect of calcium in quickening " accommodation," *i.e.*, in diminishing $\lambda$.

For very short times equation (3) gives, for threshold excitation, $U_0 - V_0 = bQ$. For very long times $U_0 - V_0 = bkI_0$, where $I_0$ is the " true rheobase." Hence $bkI_0 = bQ$, or $k = Q/I_0$. To obtain $k$ from Q and $I_1$, the " observed rheobase," $I_0$ must be calculated from $I_1$ by equation (14).

With sub-threshold constant currents the excitability changes are obtained by subtracting equations (12) and (13). We obtain,

$$U - V = U_0 - V_0 - \frac{bkI}{1 - k/\lambda}\left[e^{-t/\lambda} - e^{-t/k}\right]$$

$$= (U_0 - V_0)\left[1 - \frac{I/I_0}{1 - k/\lambda}\left(e^{-t/\lambda} - e^{-t/k}\right)\right]. \qquad (16)$$

If a condenser discharge, or induction shock, of very short duration, be employed for testing the state of the nerve at any time after turning on the constant current, its threshold strength is a measure of (U − V). The strength required falls to a minimum and then returns to its original value* along a double exponential curve, as shown in the curve XABCY of fig. 8. Bishop (1928), Erlanger and Blair (1931 *b*), and Rushton (1932) have examined this case, and Solandt will discuss it further in a later paper (1936).

In a recent monograph Bonnardel (1934) describes experiments made with "*ondes à deux échelons*," that is to say with constant currents superimposed on one another at any desired interval. In principle the object was the same as that of the experiments just referred to, to test by the second current the state of the nerve as affected by the first current. In practice such experiments are difficult and complicated to interpret. There is no doubt, in general, that the curves given by Bonnardel depend upon " accommodation " setting in, in the nerve, as the result of the first current: the second current, however, produces further " accommodation " before it succeeds in exciting, which adds on to that of the first, and moreover the state of the nerve is tested at some unknown moment after applying the second current. It is simpler and better to use condenser discharges, or induction shocks, of very short duration, to which neither of these objections applies.

## VII—CONSTANT CURRENT PULSE: " MAKE " EXCITATION

A constant current pulse of intensity I and duration $x$ can be regarded as the resultant of a constant current of intensity I starting at time zero and a constant current of intensity − I starting at time $x$. This allows us immediately, employing equations (12) and (13), to deduce the values of V and U at any time greater than $x$: for example, for V, by subtraction,

$$V = V_0 + bkI\,(e^{-(t-x)/k} - e^{-t/k}). \qquad (12')$$

The changes of excitability *during* the passage of a constant current are given by equation (16); *after* a constant current pulse of duration $x$, by subtraction,

$$U - V = U_0 - V_0 - \frac{bkI}{1 - k/\lambda}\,[e^{-(t-x)/k} - e^{-t/k} - e^{-(t-x)/\lambda} + e^{-t/\lambda}]. \qquad (16')$$

---

* In practice, under most conditions, owing to electrotonic changes of excitability (" Pflüger's law "), it will return to a somewhat different value: but the general effect s manifest.

For the same case as fig. 7, and for a current just 2% below rheobase, (U − V) is plotted in fig. 8 from equation (16′) for the three cases $x = 1$, 3, and 6 m sec respectively. The later rise of (U − V) above its original level is greater, the greater the duration of the pulse. To use Erlanger and Blair's term, the "post-cathodal depression" is greater, the greater the duration of the sub-threshold current which caused it: this is in fact what they found (1931, *a*). An experimental study of the changes of (U − V) after a constant current pulse of given duration will be given in a later paper by Solandt.

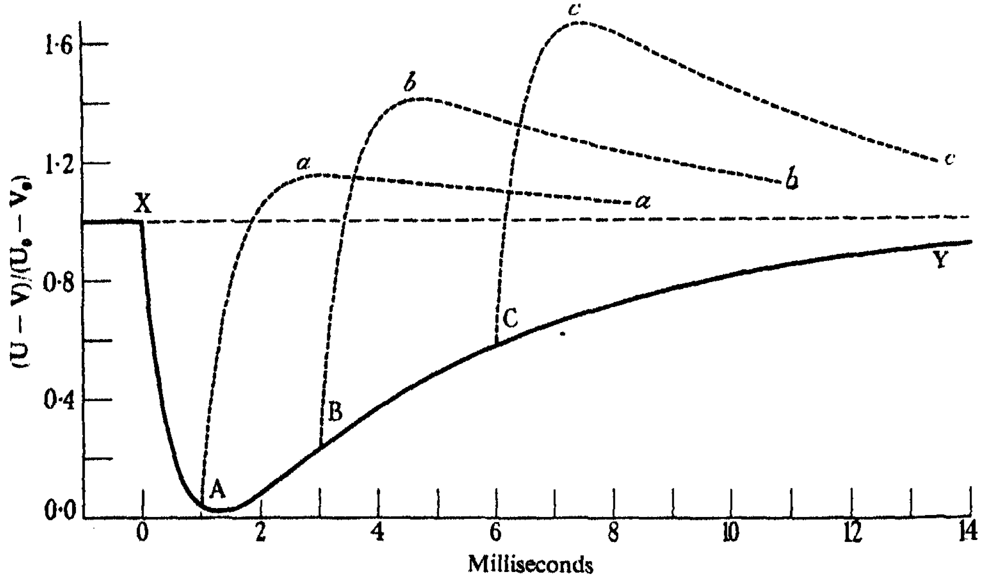


FIG. 8—Changes in (U − V) during the passage of constant currents of various durations. Calculated (as fig. 7) for frog's nerve at 15° C, very rapid "accommodation," $k = 0{\cdot}5$, $\lambda = 5$ m sec, current 98% of rheobase. Compare fig. 7 for "instantaneous" discharge. XABCY for current of long duration: XA*aa* for current lasting 1 m sec: XAB*bb* for current lasting 3 m sec: XABC*cc* for current lasting 6 m sec.

From equations (12) and (13) we can deduce the strength-duration curve for constant current pulses. Putting $t = x$ and U = V and simplifying, and remembering that $(U_0 - V_0) = bkI_0$, where $I_0$ is the "true rheobase," we obtain

$$I = \frac{I_0 (1 - k/\lambda)}{e^{-x/\lambda} - e^{x/k}}, \qquad (17)$$

or

$$I = \frac{I_1 (1 - k/\lambda)/(\lambda/k)^{\frac{1}{\lambda/k - 1}}}{e^{-x/\lambda} - e^{-x/k}}, \qquad (17')$$

where $I_1$ is the observed rheobase.

For very slow accommodation ($\lambda = \infty$) equation (17) reduces to

$$I = I_0/(1 - e^{-x/k}), \tag{18}$$

$I_0$ and $I_1$ now being equal. This well-known equation has been proposed many times for the strength-duration curve. It often fits very well. When it does not, either " accommodation," or the experimental difficulty of obtaining constant current pulses of accurately known duration, is probably the cause. For the medullated nerve of the frog, the corresponding equation for condenser discharges usually fits the facts so accurately that it seems unlikely that equation (18) is seriously at fault, except for rapid " accommodation." The matter will be discussed in a following paper.

It is interesting to compare equations (17′) and (18) numerically, (i) for a usual value of $\lambda$, and (ii) for a small value (rapid " accommodation "). The " observed rheobase," in every case, is called unity, so that the shapes of the strength-duration curves are compared *as they would be observed experimentally*. The cases taken are for a frog's nerve at 15° C, with $k = 0{\cdot}5$ m sec, and $\lambda = 5$ m sec, 25 m sec, and $\infty$ respectively.

TABLE II

| $x$: m sec .. | 0·01 | 0·02 | 0·04 | 0·08 | 0·16 | 0·32 | 0·64 | 1·28 | 2 | 3 |
|---|---|---|---|---|---|---|---|---|---|---|
| $I/I_1 : \lambda = 5$ | 39·2 | 19·8 | 10·1 | 5·29 | 2·88 | 1·70 | 1·16 | 1·00 | — | — |
| $I/I_1 : \lambda = 25$ | 46·6 | 23·6 | 12·0 | 6·25 | 3·38 | 1·97 | 1·30 | 1·04 | 1·00 | — |
| $I/I_1 : \lambda = \infty$ | 50·5 | 25·5 | 13·0 | 6·77 | 3·65 | 2·12 | 1·37 | 1·085 | 1·018 | 1·002 |

There are clearly considerable differences, particularly at short times. Actually the differences are really at long times, but they are disguised by making the " observed rheobase " unity in each case. If the numbers are plotted and the " chronaxie " read off as the time for double the observed rheobase, the following values are obtained:—

For $\lambda = \infty$, " chronaxie " = 0·347 m sec.

For $\lambda = 25$ m sec, " chronaxie " = 0·309 m sec.

For $\lambda = 5$ m sec, " chronaxie " = 0·254 m sec.

These three different values are actually for the same value of $k$, and we see how even moderate " accommodation " may cause an appreciable error (in this case 11%) in the observed " chronaxie." Rapid " accommodation " gave an apparent chronaxie 27% too low.

If the numbers for $\lambda = 25$ and for $\lambda = 5$ m sec in Table II are plotted (as described in a following paper) and a $k$ chosen to give the best possible agreement with equation (18), it is found that, except for one point ($x = 0\cdot64$ m sec for $\lambda = 5$), the agreement is comparatively good; and at short times the points converge to a line of constant quantity, as they should. The values of $k$, however, required to give this agreement are, like the "chronaxies," false. Instead of $k = 0\cdot50$, for which the numbers were calculated, we obtain:—

For $\lambda = 25$ m sec, $k = 0\cdot442$ m sec.

For $\lambda = 5$ m sec, $k = 0\cdot363$ m sec.

It would seem that a disregard of the time-factor in "accommodation" must make all "chronaxies," as ordinarily measured, "pseudochronaxies," unless the "accommodation" is very slow. We see also that an increase in Ca-ion concentration, by reducing the value of $\lambda$, may cause an apparent diminution of "chronaxie" (*cf.* Blumenfeld, 1925).

### VIII—CONSTANT CURRENT PULSE: "BREAK" EXCITATION

The case considered in § VII is the ordinary one for "make" excitation occurring at the cathode. It is possible, however, as shown by Cluzet (1908), by Cardot and Laugier (1912, *a*, *b*), and by Laugier (1921), to construct a strength-duration curve for "break" excitation occurring at the anode, the "make" excitation being eliminated by having the cathode at a distant injured part of the nerve. The duration of the current pulse being set, its strength is adjusted until break excitation just occurs. By varying the duration and measuring the corresponding strength, a strength-duration curve is obtained which is similar in form to the ordinary one, but on a very different time-scale: it can in fact be shown that its time-constant is $\lambda$ instead of $k$.

For "break" excitation, we have to consider the anode and to reverse I: the effect of the pulse of duration $x$ is the same as that of a current $-$ I starting at time zero, on which is superimposed a current $+$ I starting at time $x$. Then $(\mathrm{V} - \mathrm{V}_0)$ and $(\mathrm{U} - \mathrm{U}_0)$ are obtained respectively, for all times subsequent to $t = x$, by the use of equations (12) and (13),

$$\mathrm{V} - \mathrm{V}_0 = -\,bk\mathrm{I}\,(1 - e^{-t/k}) + bk\mathrm{I}\,(1 - e^{-(t-x)/k}),$$

*i.e.*,

$$\mathrm{V} - \mathrm{V}_0 = -\,bk\mathrm{I}\,(e^{-(t-x)/k} - e^{-t/k}), \tag{19}$$

and

$$\mathrm{U} - \mathrm{U}_0 = -\,bk\mathrm{I}\left(1 + \frac{e^{-t/k}}{\lambda/k - 1} - \frac{e^{-t/\lambda}}{1 - k/\lambda}\right)$$
$$+\,bk\mathrm{I}\left(1 + \frac{e^{-(t-x)/k}}{\lambda/k - 1} \quad \frac{e^{-(t-x)/\lambda}}{1 - k/\lambda}\right)$$

*i.e.*,

$$\mathrm{U} - \mathrm{U}_0 = -\,bk\mathrm{I}\left[\frac{e^{-(t-x)/\lambda} - e^{-t/\lambda}}{1 - k/\lambda} - \frac{e^{-(t-x)/k} - e^{-t/k}}{\lambda/k - 1}\right]. \tag{20}$$

Excitation takes place, not at $t = x$ as in " make " excitation by constant current pulse, but later, and the conditions for critical excitation are, as before, $\mathrm{V} = \mathrm{U}$ and $d\mathrm{V}/dt = d\mathrm{U}/dt$. Applying these conditions to equations (19) and (20) and simplifying we obtain,

$$\frac{bk\mathrm{I}}{\mathrm{U}_0 - \mathrm{V}_0} = \left(\frac{\lambda}{k}\right)^{\frac{1}{\lambda/k-1}} \frac{(1 - e^{-x/k})^{\frac{1}{\lambda/k-1}}}{(1 - e^{-x/\lambda})^{\frac{1}{1-k/\lambda}}}.$$

Now for all values of $x$ greater than (say) $2k$, and for any reasonable value of $\lambda$, $(1 - e^{-x/k})^{\frac{1}{\lambda/k-1}}$ is effectively unity. For example if $x = 2k$ and $\lambda/k = 40$ it is 0·996, for $\lambda/k = 20$ it is 0·992, for $\lambda/k = 10$ (an extreme case) it is 0·984. For any case, therefore, in which we are likely to be interested we may write (putting $\mathrm{U}_0 - \mathrm{V}_0 = bk\mathrm{I}_0$)

$$\mathrm{I} = \frac{\mathrm{I}_0\left(\frac{\lambda}{k}\right)^{\frac{1}{\lambda/k-1}}}{(1 - e^{-x/\lambda})^{\frac{1}{1-k/\lambda}}}. \tag{21}$$

Since $1/(1 - k/\lambda)$ is usually about 1·02 and never greater than about 1·10 we can, for a first approximation, write,

$$\mathrm{I} = \frac{\text{constant}}{1 - e^{-x/\lambda}}. \tag{21$'$}$$

This equation, applied to the data in the paper by Cardot and Laugier (1912, *a*), gives a reasonably good fit at the longer times, with $\lambda = 5{\cdot}8$ m sec, which though small (rapid " accommodation ") is within the normal range. Laugier (1921) gives a number of experiments of this type, from which it appears (i) that heart muscle has very slow " accommodation," and (ii) that " accommodation " is quickened by a rise of temperature. The matter will be discussed more fully by Solandt in a later paper (1936), where the values of $\lambda$ obtained by this and other methods will be compared.

## IX—GAP IN CONSTANT CURRENT

A gap of duration $x$ in a constant current of strength I may excite in two ways, just as may a constant current pulse (*see* §§ VII and VIII) viz.:—

(1) at the anode after breaking the current;
(2) at the cathode after remaking it.

Strength-duration curves may be constructed for both: they are similar in form, but of different time relations: the principal time-constant of process (1) is $k$, that of process (2) is $\lambda$.

We suppose that the nerve is completely "accommodated" to the current before the gap is applied. Then for case (1) at the anode, equations (12) and (13) give, at $t = \infty$,

$$\mathrm{V} - \mathrm{V}_0 = - bk\mathrm{I}$$
$$\mathrm{U} - \mathrm{U}_0 = - bk\mathrm{I}.$$

Breaking the current is equivalent to making a current + I in the opposite direction, which at time $x$ thereafter would have the further effect,

$$\mathrm{V} - \mathrm{V}_0 = bk\mathrm{I}\,(1 - e^{x/k})$$
$$\mathrm{U} - \mathrm{U}_0 = bk\mathrm{I}\left(1 + \frac{e^{-x/k}}{\lambda/k - 1} - \frac{e^{-x/\lambda}}{1 - k/\lambda}\right).$$

The total effect of a gap of duration $x$ is therefore, by addition,

$$\mathrm{V} - \mathrm{V}_0 = - bk\mathrm{I}e^{-x/k},$$
$$\mathrm{U} - \mathrm{U}_0 = - bk\mathrm{I}\left[\frac{e^{-x/\lambda}}{1 - k/\lambda} - \frac{e^{-x/k}}{\lambda/k - 1}\right].$$

If excitation occurs at time $x$ then $\mathrm{V} = \mathrm{U}$, and remembering that $\mathrm{U}_0 - \mathrm{V}_0 = bk\mathrm{I}_0$ we obtain,

$$\mathrm{I} = \frac{\mathrm{I}_0\,(1 - k/\lambda)}{e^{-x/\lambda} - e^{-x/k}}.$$

This is identical with equation (17) above, which represents the effect at the cathode of a constant current pulse; its principal time-constant is $k$.

Lucas (1907, *a*) investigated case (2); he stated "the current was a descending one, so that make alone was effective, while break alone was ineffective." In this case excitation was at the cathode and the sequence of events may be regarded as follows:

(*a*) a constant current I passed till "accommodation" was complete;
(*b*) a current − I passed from time $t = 0$ onwards;
(*c*) a current + I passed from time $t = x$ onwards.

The net effect can be obtained by addition, as before:

$$\mathrm{V} - \mathrm{V}_0 = bk\mathrm{I}\,(1 - e^{-(t-x)/k} + e^{-t/k})$$
$$\mathrm{U} - \mathrm{U}_0 = bk\mathrm{I}\left[1 + \frac{e^{-(t-x)/k} - e^{-t/k}}{\lambda/k - 1} - \frac{e^{-(t-x)/\lambda} - e^{-t/\lambda}}{1 - k/\lambda}\right].$$

These equations are identical with equations (19) and (20) above, except that *bk*I is added to the right-hand side in each case. For threshold excitation, therefore, the solution is the same as for those, viz., equations (21) and (21′) above. Lucas's results are fitted rather accurately by the simplified form (21′) as the calculations given in Table III show.

TABLE III

*Experiment* 1—8·1° C. Observed rheobase $I_1 = 1 \cdot 4$ μA. λ used for calculation = 15 m sec

| | | | | | |
|---|---|---|---|---|---|
| Time, m sec .............. | ∞ | 17 | 8·7 | 5·2 | 2·6 |
| $I/I_1$ observed .............. | 1 | 1·52 | 2·13 | 3·55 | 6·2 |
| $I/I_1$ calculated ............ | 1 | 1·48 | 2·27 | 3·42 | 6·2 |

*Experiment* 2—8·1° C. Observed rheobase 1·3 μA. λ used for calculation = 10 m sec

| | | | | | |
|---|---|---|---|---|---|
| Time, m sec .............. | ∞ | 17 | 8·7 | 5·2 | 2·6 |
| $I/I_1$ observed .............. | 1 | 1·34 | 1·57 | 2·55 | 4·98 |
| $I/I_1$ calculated ............ | 1 | 1·22 | 1·72 | 2·46 | 4·37 |

The values of λ calculated from Lucas's two experiments, 15 and 10 m sec respectively, are rather small (" accommodation " rapid) but well within the range found by other methods. Lucas used a high Ca-ion concentration in his Ringer's fluid, which tends to give small values of λ. Further experiments on the subject will be described by Solandt (1936).

The following tabular statement may be given of §§ VII, VIII, and IX.

| Nature of current | Excitation at | Electrode | Principal time-constant |
|---|---|---|---|
| Pulse | Make | Cathode | *k* |
| | Break | Anode | λ |
| Gap | Break | Anode | *k* |
| | Remake | Cathode | λ |

The simplest way of regarding a gap is as a pulse in the opposite direction, superimposed on the steady current: in which case the phenomena for the gap for the fully accommodated nerve, are the same as those for the pulse.

## X—LINEARLY INCREASING CURRENT

Keith Lucas (1907, *b*, 1908) following von Kries (1884, *b*, *c*) used a mechanical arrangement for increasing the current at a uniform rate up to a certain value, at which it then remained constant. Fabre (1927, etc.) employed "*ondes cunéiformes*," produced electrically, *i.e.*, uniformly increasing currents cut short abruptly at a given value or time. Both determined the relation between the threshold strength of current finally attained and the time taken in attaining it: their relations are similar at long but different at short times. Lucas found a "minimal current gradient" ($(dI/dt)/I_0$) to exist, below which no excitation is possible. Fabre took the reciprocal of the minimal current gradient as a time-constant characteristic of the nerve: he gave it the name "*constante linéaire*" or C.L. Actually Fabre's C.L. is the same as $\lambda$, the time-constant of "accommodation": it has no special connexion with linear currents.

Let $I/I_0 = \mu\theta$, where $I_0$ is the "true rheobase," $\theta$ is time and $\mu$ is the current gradient. Substituting $I_0\mu\theta$ for $I_\theta$ in equation (4) we find,

$$V - V_0 = I_0\,\mu bk\,[t - k\,(1 - e^{-t/k})]. \tag{22}$$

Inserting this for $(V_\theta - V_0)$ in equation (7) we obtain

$$U - U_0 = I_0\mu bk\left[t - (\lambda + k) + \frac{\lambda^2}{\lambda - k}\,e^{-t/\lambda} - \frac{k^2}{\lambda - k}\,e^{-t/k}\right]. \tag{23}$$

Excitation will occur as soon as V becomes equal to U. Putting $V = U$ and remembering that $U_0 - V_0 = bkI_0$ we obtain,

$$\mu = \frac{1}{\lambda\left(1 - \dfrac{\lambda}{\lambda - k}\,e^{-t/\lambda} + \dfrac{k}{\lambda - k}\,e^{-t/k}\right)}. \tag{24}$$

This gives the current gradient $\mu$ required to effect excitation at time $t$. The maximum value of the denominator (at $t = \infty$) is $\lambda$, so a minimal current gradient exists below which no excitation at all can be effected. The value of the minimum current gradient is $1/\lambda$, which shows that $\lambda$ is the same as Fabre's "*cpnstante linéaire.*"

The relation between the current I at which excitation is finally attained and the time $t$ taken to attain it is given by putting $I/I_0 = \mu t$ in equation (24); it is,

$$I = \frac{I_0 t}{\lambda\left(1 - \dfrac{\lambda}{\lambda - k}\,e^{-t/\lambda} + \dfrac{k}{\lambda - k}\,e^{-t/k}\right)}. \tag{24'}$$

Fabre (1927, *a*) gives an experiment with "*ondes cunéiformes*" from which, in fig. 9*a*, I is plotted against $t$. In fig. 9*b*, a curve calculated from equation (24′) is shown for comparison. Although the curves

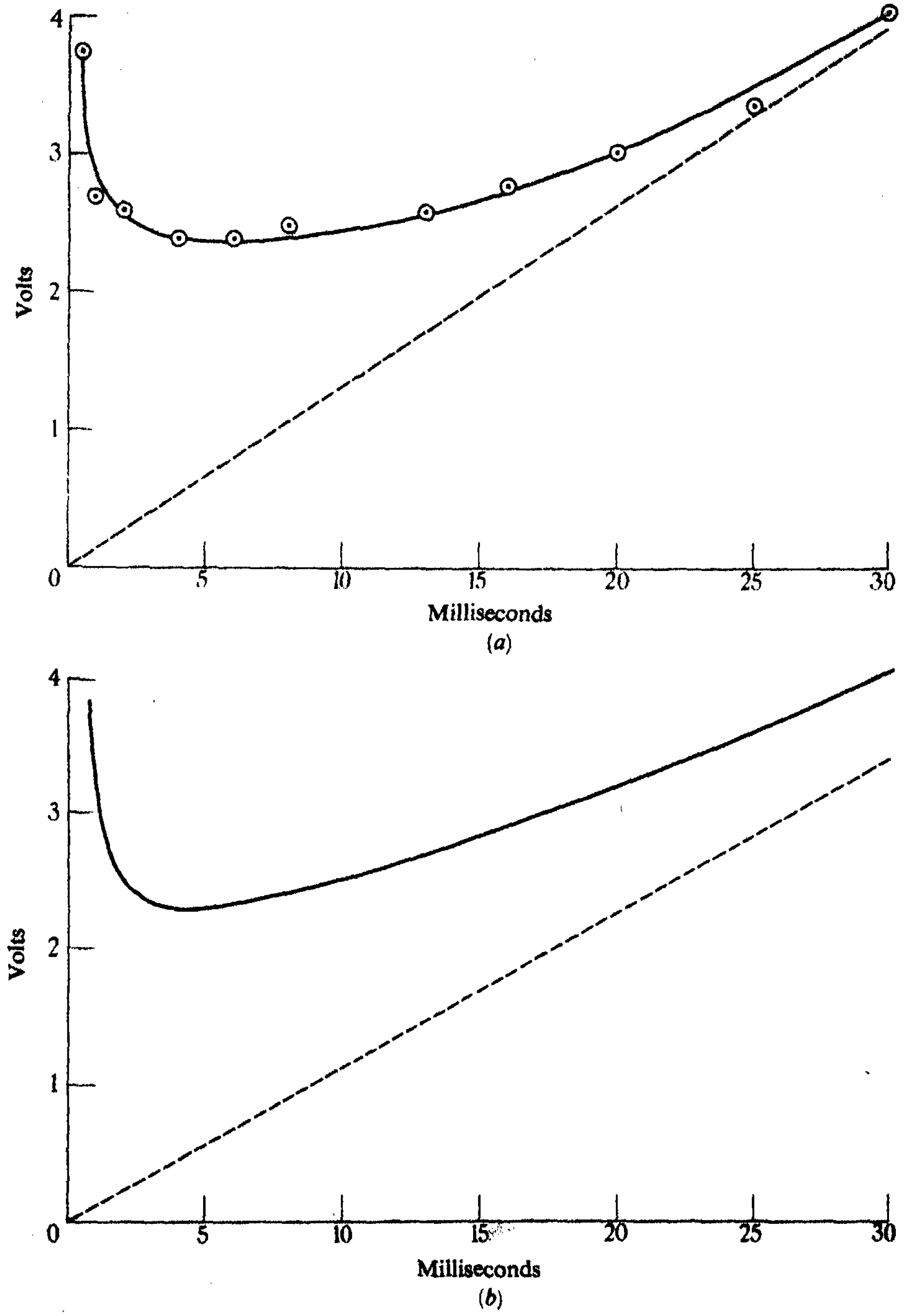


FIG. 9—Linear progressive currents (*ondes cunéiformes*). Abscissa, duration of current increase: ordinate, threshold voltage. (Current cut off at end of increase). (*a*) Fabre's experimental results: 20° C: observed rheobase 2 volts: observed "chronaxie" 0·33 m sec ($k$ = 0·476 m sec): observed "*constante linéaire*" 15 m sec. (*b*) Curve calculated from equation (24′) for true rheobase 1·8 volts, $k$ = 0·5 m sec., $\lambda$ = 16 m sec.

are not identical they are so similar that no doubt can exist that equation (24′) correctly expresses the general relation of the factors involved in linear progressive currents.

In Lucas's (1907, *b*, 1908) experiments the current was not cut off when it had reached its full value, but remained constant. At the shorter times excitation must have occurred during the constant and not during the rising phase. This makes the calculation of a theoretical curve more complicated. His " minimal current gradients," however, are easy to interpret. In the toad's sciatic at 10° to 11·5° C Lucas found a minimal current gradient of about 46, *i.e.*, $\lambda = 22$ m sec: in the frog's sciatic at 10° to 11·5° C a minimal gradient of about 63, *i.e.*, $\lambda = 16$ m sec: in the toad's sartorius at 12° to 15·5° C a minimal current gradient of about 4·4, *i.e.*, $\lambda = 230$ m sec. The values for nerve are well within the norma range reported by Solandt in the following paper. Lucas (1908, p. 473) also found that the minimal gradient is very low (" accommodation " slow) in a muscle bathed in a Ca-free solution, more rapid when calcium is admitted, and from the progressive change noticed in excised muscles kept in Ringer's fluid he suggested that the concentration of Ca he used (0·024% $CaCl_2$) is abnormally high. The effect of Ca on the speed of " accommodation " in muscle was confirmed by Kahn (1911). For nerve the matter has been more fully investigated by Solandt.

From equations (22) and (23) by subtraction we obtain, putting $I_0bk = U_0 - V_0$,

$$U - V = (U_0 - V_0)[1 - \mu\lambda\{1 - \lambda e^{-t/\lambda}/(\lambda - k) + ke^{-t/k}/(\lambda - k)\}]. \tag{24''}$$

This could be compared with Fabre's (1934, *b*) results on the changes of excitability during the passage of a linearly increasing current.

## XI—Exponentially Increasing Currents

For making exponentially increasing currents of the form

$$I = I_2(1 - e^{-t/a}),$$

where $I_2$ is the final value of I at $t = \infty$ and $a$ is the time-constant of rise of I, two methods are available, viz., that employing an inductance (Gildemeister, 1904, 1913) and that employing a condenser with resistances (Lapicque, 1908, *a*, 1926; Cardot and Laugier, 1913; Delville, 1934). The latter is much the easier and has been employed in the present investigation, as described in the following paper by Solandt. It has a wide range, and, provided that good non-polarizable electrodes are used (a precaution not always taken), it gives very accurate results.

Substituting for I in equation (4) above we obtain,

$$\mathrm{V} - \mathrm{V}_0 = bk\mathrm{I}_2\left[1 - \frac{e^{-t/k}}{1 - a/k} - \frac{e^{-t/a}}{1 - k/a}\right]. \tag{25}$$

Inserting this in equation (7) we find,

$$\mathrm{U} - \mathrm{U}_0 = bk\mathrm{I}_2\left[1 - \frac{e^{-t/k}}{(1 - \lambda/k)(1 - a/k)} - \frac{e^{-t/a}}{(1 - \lambda/a)(1 - k/a)} - \frac{e^{-t/\lambda}}{(1 - k/\lambda)(1 - a/\lambda)}\right]. \tag{26}$$

The conditions for threshold excitation are, as usual, $\mathrm{V} = \mathrm{U}$ and $d\mathrm{V}/dt = d\mathrm{U}/dt$. These conditions, applied to equations (25) and (26) as they stand, lead to very complex results. In dealing experimentally, however, with the subject it is always found that $k$ is small compared with $\lambda$, and that the times in which we are interested are so much greater than $k$ that the terms in $e^{-t/k}$ (the denominators also of which are large) can be neglected. With the provision that the utilization time (referred to below) is not less than (say) $5k$, this involves no significant error. We then have,

$$\mathrm{V} - \mathrm{V}_0 = bk\mathrm{I}_2\left[1 - \frac{e^{-t/a}}{1 - k/a}\right], \tag{25'}$$

and

$$\mathrm{U} - \mathrm{U}_0 = bk\mathrm{I}_2\left[1 - \frac{e^{-t/a}}{(1 - \lambda/a)(1 - k/a)} - \frac{e^{-t/\lambda}}{(1 - a/\lambda)(1 - k/\lambda)}\right]. \tag{26'}$$

Applying the condition $d\mathrm{V}/dt = d\mathrm{U}/dt$ for threshold excitation we find, for the utilization time $t_1$,

$$t_1/a = \frac{\log_e\left(\dfrac{a - k}{\lambda - k}\right)}{a/\lambda - 1}, \tag{27}$$

which, if $k$ is small compared with $a$ and with $\lambda$, becomes

$$(t_1 - k)/a = \frac{\log_e(a/\lambda)}{a/\lambda - 1}. \tag{27'}$$

Equations (27) and (27′) allow us to calculate the utilization time for an exponentially increasing current, a matter to which research has frequently been devoted (*e.g.*, Gildemeister, 1913; Lapicque, 1926, p. 137;

Suzuki, 1932). Putting U = V and inserting the value of $t_1$, simplifying, and putting $U_0 - V_0 = bkI_0$, we obtain,

$$I_2/I_0 = (1 - k/\lambda)\left(\frac{a-k}{\lambda-k}\right)^{\frac{a/\lambda}{a/\lambda-1}}. \tag{28}$$

If $k/a$ and $k/\lambda$ are small, so that their squares can be neglected, this finally becomes,

$$I_2/I_0 = (a/\lambda)^{\frac{a/\lambda}{a/\lambda-1}}. \tag{28'}$$

Provided that $t_1$ is not less than (say) $5k$, equations (25′), (26′), (27) and (28) are very nearly exact: equations (27′) and (28′) are close approximations provided further that $k/a$ is not too large ($k/\lambda$ is always small). Equation (28′) is the one required for the experimental determination of $\lambda$; for its correct use $a$ should not be less than about $10k$, *i.e.*, for a frog's nerve at 15° C about 5 m sec. From this value of $a$ upwards, equations (27′) and (28′) are sufficiently exact.

In equation (28′) $I_2$ is the final value of the current, $a$ the time-constant of its rise, and $I_0$ the "true rheobase." In applying it we must avoid values of $a$ less than about $10k$—otherwise we must use equation (28). The numbers in Table IV allow equation (28′) to be plotted, as in fig. 10.

TABLE IV

| $a/\lambda$ ...... | 0·5 | 1 | 2 | 3 | 4 | 5 | 7 | 10 | 14 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|
| $I_2/I_0$ ...... | 2 | 2·72 | 4 | 5·2 | 6·35 | 7·47 | 9·7 | 12·9 | 17·1 | 23·4 |

The theoretical relation is approximately linear over the whole range in which it can properly be applied. Its final slope is 1 : 1. Hence if $I_2/I_0$ is plotted against $a$, the final slope is $1/\lambda$. This affords an experimental means of finding $\lambda$. The linearity of the relation has been confirmed by Solandt (1935) in experiments which will be further described in the following paper. He used calomel half-cells as electrodes. Values of the slope from 9 to 140 were found for frog's nerve at room temperature, *i.e.*, values of $\lambda$ from 110 to 7 m sec. Delville (1934) reports a number of experiments by a similar method: an analysis of his tables shows that when he did not use a shunt to his silver electrodes (which causes exaggerated polarization) he obtained a good linear relation. The values given in Table V have been calculated from his results.

TABLE V

| **Table** ............ | 1 | 2 | 3 | 4 | 9 | 10 | 12 | 16 | **Mean** |
|---|---|---|---|---|---|---|---|---|---|
| **$\lambda$: m sec** .......... | 7·5 | 6·8 | 7·9 | 5·6 | 5·5 | 4·2 | 7·6 | 8·0 | 6·6 |

The mean value is small (rapid " accommodation "). Possibly Delville used a high concentration of Ca; possibly the silver electrodes had something to do with it.

Cardot and Laugier (1913), employing non-polarizable (Zn-$ZnSO_4$) electrodes, found " *une loi sensiblement linéaire* " between the final value of the current and the time-constant of its rise, thus also confirming equation (28′). It is remarkable that, on general grounds, they concluded that " *la pente de cette droite est la pente limite*," in other words that the slope of the relation between threshold strength and time-constant of rise of exponential current is the same as the " minimal

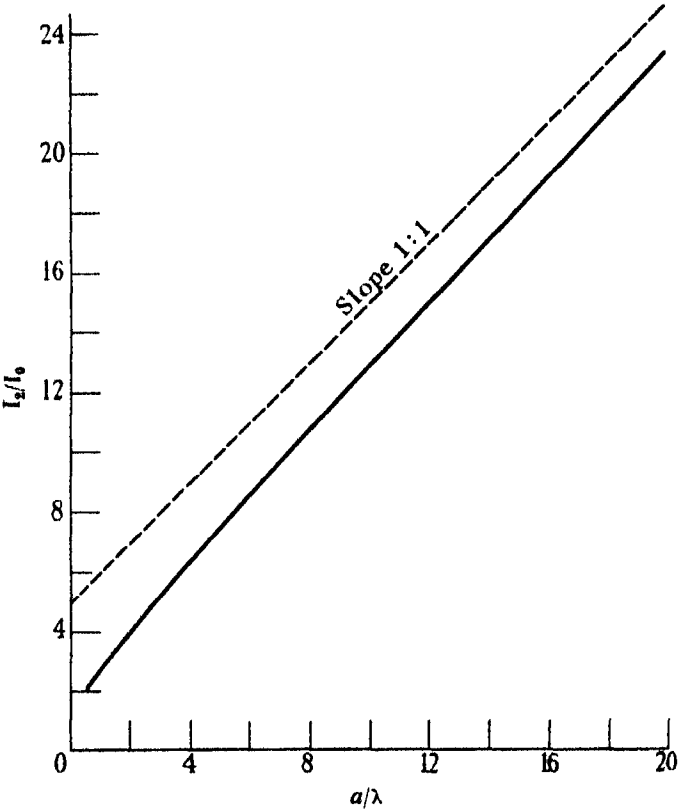


FIG. 10—Threshold excitation by exponentially increasing current, equation (28′). Vertically, final current strength as a multiple of " true rheobase." Horizontally, time-constant of rise as a multiple of time-constant of " accommodation." Broken line at slope of 1 : 1, showing that the graph of equation (28′) rather rapidly converges to that slope.

gradient" *for linear current: it has been shown above that both are* $1/\lambda$. Three experiments on a gastrocnemius-sciatic preparation from *Rana fusca* (February) gave excellent straight lines of slopes 95, 121, and 62 respectively ($\lambda = 10\cdot5$, $8\cdot3$, and $16\cdot1$ m sec). Laugier (1921) records two further experiments on frog's nerve, but these were made with silver electrodes and the lines are badly curved, probably owing to polarization. "Accommodation" in the ventricle of the frog was very slow; in two experiments λ was 0·5 sec and 2·0 sec respectively, in snail's heart it was practically infinite.

Schriever (1930), with exponential currents, employing the condenser method and non-polarizable (porcelain-boot) electrodes, found a striking linearity of the relation between $I_2$ and $a$. The values of λ deduced from the slopes of his lines relating $I_2/I_1$ to $a$ are given in Table VI.

TABLE VI

| Experiment No. . . | 1 | 2 | 3 | 4 | 5 | 9 | 10 | 11 | 12 | 15 | 16 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| λ: m sec . . . . . . . . | 4·8 | 5·3 | 6·4 | 6·3 | 4·7 | 4·6 | 4·0 | 6·1 | 2·9 | 37 | 45 |

Experiments 1–5 were on frogs' sciatics at 18–23° C. Experiments 9–12 were on toads' sciatics at 23–26° C. Experiments 15 and 16 were on the motor point of the biceps in man. The last two are in good agreement with Solandt's (1935) results. The others are very short.

In a second paper (1932, *a*) Schriever went into the matter more closely and, on the analogy of the "chronaxie," defined a time-parameter of "accommodation," or as he called it, of "*Einschleichen von Strom.*" He first took the rheobase $I_1$ with constant current of instantaneous increase: then for $I_2 = 2I_1$ he found the value of $a$ at which excitation just occurred. The "*Halbzeit*" for this current ($= 0\cdot693a$) he defined as the "*Einschleichzeit*," or as the "*chronherpie.*" Neglecting, for simplicity, the difference between $I_1$ and $I_0$ (the observed and the true rheobase—*see below*) equation (28′) above and Table IV show that for $I_2/I_0 = 2$, $a/\lambda = 0\cdot5$. Thus $\lambda = 2a$, or $\lambda = 2/0\cdot693 = 2\cdot89 \times$ (the "*Einschleichzeit*"). From this the absolute values of λ in his tables can be calculated.

For frogs' sciatics at room temperature the "*Einschleichzeit*" varied usually from 3 to 5 m sec, with an average of about 4 m sec. These correspond to $\lambda = 9$ to 14 m sec, with an average of about 11½ m sec. Lucas's results (§ X), at 10–11·5° C give an average of about 16 m sec; with an ordinary temperature coefficient the agreement is good. Both, however, are small compared with Solandt's figures, but perhaps Schriever, like Lucas, used a high Ca-concentration in his Ringer's fluid. For toads, $\lambda$ calculated from Schriever's results averaged about 10 m sec. Lucas's figures give 22 m sec at 10° to 11·5° C. For man, $\lambda$ from Schriever results varied from 37 to 92 m sec, with an average (for six persons) of 68 m sec: this agrees well with Solandt (*see* the following paper).

The ratio $\lambda/k$ is twice that of "*Einschleichzeit*" to "chronaxie," since the former is $\lambda/2{\cdot}89$, the latter $k/1{\cdot}44$ (*see* § XII below). Schriever determined both "*Einschleichzeit*" and "chronaxie" in a number of experiments; from his results an average value of $\lambda/k$ of about 30 can be calculated. This is well within the normal range found in the present investigation. Schriever varied several conditions (resistances of circuit, distance between electrodes, temperature) and found always that $k$ and $\lambda$ altered in the same direction: his range of $\lambda/k$ is comparatively narrow. Apparently he never altered the Ca-ion concentration of his Ringer's fluid: had he done so he would have found, as Solandt has found, a wide variation of $\lambda/k$.

In one preparation Schriever varied the temperature from 20·5 to 37° C: $\lambda$ calculated from his figures changed from 19 m sec to 4 m sec. In another preparation the temperature was altered from 20° to 4·5° C: $\lambda$ changed from 26 m sec to 123 m sec. The effect of temperature on the speed of "accommodation" is a normal one for biological reactions. The data, however, are not sufficient to calculate a temperature coefficient.

In a third paper (1932, *b*) Schriever discussed the nature of what we may now call "accommodation," in terms of a polarization model.

A few observations of the relation between strength $I_2$ and time-constant $a$ of rise, for an exponential current applied to human nerve, have been given by Liberson (1934).

Gildemeister (1913) reports a few experiments with exponential currents, but they are chiefly concerned with the utilization time. He showed that the latter is diminished by Ca: this is the usual effect of Ca on $\lambda$. His results could be compared with equation (27′).

Equations (28) and (28′) contain $I_2/I_0$, where $I_0$ is the "true rheobase." In experimental work the "observed rheobase" $I_1$ will be used, and $I_2/I_1$, instead of $I_2/I_0$ will be plotted against $a$. Since $I_1$ is greater than $I_0$ the resulting slope will be too small, sometimes seriously as Table I shows. From equation (14), however, $I_1/I_0$ can be calculated, and so correction made. This may involve a process of successive approximation in finding the true value of $\lambda$, but the labour involved is not great.

During the development of an exponentially increasing current the value of $(U - V)$, and hence the intensity of a short shock just strong enough to excite, first falls, then reaches a minimum, then rises again. Subtracting equations (25′) and (26′),

$$U - V = (U_0 - V_0)\left[1 - \frac{I_2/I_0}{1 - a/\lambda}\left(\frac{e^{-t/\lambda}}{1 - k/\lambda} - \frac{e^{-t/a}}{1-k/a}\right)\right]. \qquad (29)$$

This equation was employed in calculating the broken curve of fig. 6*a*.

### XII—Condenser Discharges

When a condenser of capacity C, charged initially to potential E, is discharged through a resistance R, the current I at time $\theta$ is $I = Ee^{-\theta/RC}/R$. Inserting this in equation (4) we obtain,

$$V - V_0 = \frac{bkE/R}{1 - k/RC}\left(e^{-t/RC} - e^{-t/k}\right). \qquad (30)$$

V rises to a maximum and falls again: the maximum is at a time $t_1$ given by,

$$\frac{t_1}{RC} = \frac{\log_e (RC/k)}{RC/k - 1}. \qquad (31)$$

Neglecting "accommodation," $t_1$ is the utilization time.

If threshold excitation is to occur (neglecting "accommodation"), the maximum value of V must be equal to $U_0$. Let $E_0$ be the rheobasic potential, *i.e.*, $E_0/R = I_0$. Then $U_0 - V_0 = bkI_0 = bkE_0/R$, and the condition for threshold excitation becomes

$$E/E_0 = (RC/k)^{\frac{1}{RC/k-1}}. \qquad (32)$$

This is the "strength-duration curve" for condenser stimulation, strength being potential E, duration being time-constant RC of condenser discharge. It has been proposed before by Rushton (1931, p. 272), and by Blair (1932, *a*, *b*; 1934), of whom the latter has made extensive tests confirming its application to experimental data. It has been found in this laboratory to be obeyed with considerable accuracy (*cf.* Hill, 1935, *a*), as will be described in detail in a later paper (Hill, 1936).

For constant current pulses, neglecting accommodation, from equation (18) above, the duration for double the rheobase (Lapicque's "chronaxie," Lucas's "excitation time") is $0{\cdot}693k$. For condenser discharges, from equation (32), the value of RC for double the rheobase is $2k$. The ratio of these is $0{\cdot}693/2 = 0{\cdot}347$, which enables one, if one wishes, to deduce the "chronaxie" from condenser experiments. Lapicque (1926, p. 324) has made numerous and careful experimental determinations of this ratio. His approved mean value is 0·37, which is close to the theoretical one (*cf.* Eichler, 1932, 1933; Blair, 1932, *a*, *b*).

For excitation by condenser discharges, the value of RC for minimum energy is $k$. On the double logarithmic plot this is the point at which the slope of the tangent is $-\frac{1}{2}$ (Rushton, 1931). This enables us easily to find $k$ from a portion only of the curve, as *e.g.*, when the rheobase cannot be determined owing to repetitive response to long-lasting currents.

To find U we insert the value of V from equation (30) in equation (7) and obtain,

$$\mathrm{U} - \mathrm{U}_0 = \mathrm{RC}bk\mathrm{E}/\mathrm{R}\left[\frac{ke^{-t/k}}{(\mathrm{RC}-k)(\lambda-k)} + \frac{\mathrm{RC}e^{-t/\mathrm{RC}}}{(\lambda-\mathrm{RC})(k-\mathrm{RC})} + \frac{e^{-t/\lambda}}{(k-\lambda)(\mathrm{RC}-\lambda)}\right]. \quad (33)$$

From equations (30) and (33) by subtraction, and putting

$$\mathrm{U}_0 - \mathrm{V}_0 = bk\mathrm{E}_0/\mathrm{R},$$

$$\mathrm{U} - \mathrm{V} = (\mathrm{U}_0 - \mathrm{V}_0)\left[1 + \mathrm{RC}\lambda\mathrm{E}/\mathrm{E}_0\left\{\frac{e^{-t/k}}{(\mathrm{RC}-k)(\lambda-k)} + \frac{e^{-t/\mathrm{RC}}}{(k-\mathrm{RC})(\lambda-\mathrm{RC})} + \frac{e^{-t/\lambda}}{(k-\lambda)(\mathrm{RC}-\lambda)}\right\}\right]. \quad (34)$$

If we consider only times greater than $5k$, so that the term in $e^{-t/k}$ can be neglected, this becomes,

$$\mathrm{U} - \mathrm{V} = (\mathrm{U}_0 - \mathrm{V}_0)\left[1 - \frac{\mathrm{E}}{\mathrm{E}_0} \cdot \frac{\lambda\,\mathrm{RC}}{\lambda - \mathrm{RC}}\left(\frac{e^{-t/\mathrm{RC}}}{\mathrm{RC}-k} - \frac{e^{-t/\lambda}}{\lambda - k}\right)\right]. \quad (34')$$

Equations (34) and (34′) can be tested experimentally by very rapid condenser discharges applied at a series of given instants after the primary discharge. Fig. 11 illustrates the changes of excitability, during a just sub-threshold condenser discharge, calculated from equation (34) for the conditions shown in the legend. (U − V) falls rapidly to nearly zero at about 1·2 m sec; then rises again, crosses its original value, and remains high for a long period. The " post-cathodal depression " is here very obvious, as Erlanger and Blair (1931, *a*) found it to be with long condenser discharges.

The conditions for threshold excitation, $\mathrm{V} = \mathrm{U}$ and $d\mathrm{V}/dt = d\mathrm{U}/dt$, lead to very complex equations and it has proved impossible to find a simple expression for the strength-duration (voltage-capacity) relation, taking proper account of " accommodation."

"*Double Condenser Discharges*"—Lapicquè (1926, p. 129) and his colleagues (*e.g.*, Monnier, 1934, *b*, p. 115: *see also* Wyss (1934)) have examined the case of slowly rising condenser discharges of the form $I = I_2 (e^{-t/\alpha} - e^{-t/\beta})$. These show the usual effects associated with gradually increasing currents, but their theoretical analysis is complicated. They could scarcely, in principle, reveal any phenomena not already exposed by simple condenser discharges, or by exponentially rising currents: so the greater ease of interpreting the results of the latter makes them preferable. If, for any special reason, it were desired to obtain equations, similar to the above, for "double condenser discharges," one could do so by adding the effects (on $(V - V_0)$, or on $(U - U_0)$) of $I_2 e^{-t/\alpha}$ and of $-I_2 e^{-t/\beta}$: or of $I_2 (1 - e^{-t/\beta})$ and of $-I_2 (1 - e^{-t/\alpha})$: as given respectively in §§ XII and XI above.

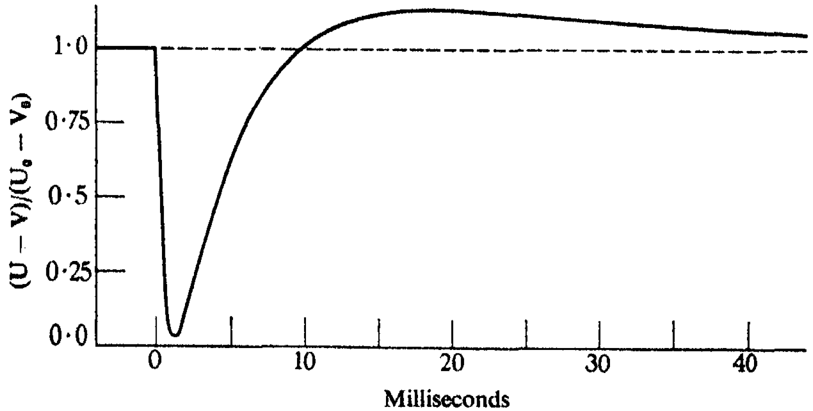


FIG. 11—Changes in (U − V) during the passage of a condenser discharge of nearly threshold strength. Calculated from equation (34) for frog's nerve at 15° C, average speed of "accommodation," $k = 0 \cdot 5$ m sec, $\lambda = 20$ m sec, $E_0 = 1$ volt, RC = 5 m sec, E = 1·3 volts, utilization time about 1·2 m sec. Compare figs. 7 and 8.

## XIII—ALTERNATING CURRENT

Put $I \sin (2\pi n\theta + \varepsilon)$ for the current in equation (4), where $\theta$ is time, $n$ is frequency, and $\varepsilon$ is phase of starting: then

$$V - V_0 = \frac{bkI}{\sqrt{1 + 4\pi^2 k^2 n^2}} \{\sin (2\pi nt + \varepsilon - \alpha) - e^{-t/k} \sin (\varepsilon - \alpha)\}, \quad (35)$$

where $\tan \alpha = 2\pi nk$.

The exponential term dies rapidly out: *e.g.*, if $k = 0 \cdot 5$ m sec (as for a frog's nerve at 15° C), $e^{-t/k}$ is effectively zero at 2 m sec: in that time, owing to the refractory period, one response only is possible. Apart, therefore, from the first few milliseconds, and the possibility of one anomalous response (depending on the phase of commencing the stimulus) we may write, disregarding the phase,

$$V - V_0 = bkI \sin 2\pi nt/\sqrt{1 + 4\pi^2k^2n^2}. \qquad (35')$$

If no account is taken of "accommodation," threshold excitation occurs if and when the maximum value of V becomes equal to $U_0$. Putting $(U_0 - V_0) = bkI_0$, where $I_0$ is the "true rheobase," we obtain

$$I/I_0 = \sqrt{1 + 4\pi^2k^2n^2}. \qquad (36)$$

This is the condition for steady threshold stimulation, once to each cycle at the electrode considered. A similar equation has been given by Blair (1932, *a*, p. 747). It may be expected to hold above the region of frequency in which "accommodation" is important, and below that in which the absolute refractory period prevents response to every cycle: in frog's nerve, therefore, at 20° C, between 150 and 1000 cycles per second. An experimental investigation of the relation between threshold strength and frequency will be given in a later paper (Hill, Katz, and Solandt, 1936).

The exponential term provides the possibility of one anomalous response, depending upon the phase (which cannot easily be controlled) at which the current through the nerve begins. Its effect in increasing the maximum value of $(V - V_0)$ is greatest if $\varepsilon = 0$, *i.e.*, if the current starts exactly at the beginning of a wave. At low frequency this effect is small; at high frequency it is greater as shown by the values given in Table VII, calculated for a frog's nerve at 15° C, with $k = 0{\cdot}5$ m sec.

TABLE VII

| | | | | | | |
|---|---|---|---|---|---|---|
| Frequency, cycles/sec........ | 106 | 318 | 636 | 1273 | 3180 | Very high |
| Maximum % increase in maximum value of $(V - V_0)$, due to exponential term ...... | 0·3 | 7 | 25 | 45 | 75 | 100 |

Thus at higher frequency there is serious danger of getting a false result, if a single twitch be taken as index of excitation: the only reliable index is a steady response.

This rapidly vanishing exponential term is almost certainly the mathematical expression of the *Anfangszuckung* referred to by von Kries (1884, *a*, p. 196). He found the latter to be similar to a single twitch, and to appear most readily at a high frequency. The exponential term occurred already in Nernst's (1908) discussion of the problem, and was further referred to by Gildemeister (1929), by Buchloh (1931) and by Blair (1932, *a*.) Coppée (1934) observed its effect experimentally, and, in order to avoid it, increased his stimulating current gradually to threshold. It is obvious physically that a half-wave beginning exactly at phase zero will raise the " local potential " higher than later half-waves, since it will not first have to neutralize the remaining effect of the preceding half-wave in the opposite direction.

To allow for " accommodation " we insert the value of $(V - V_0)$ from equation (35) in equation (7), neglecting the exponential term, and putting $\varepsilon = 0$, and finally obtain

$$U - U_0 = bkI \sin (2\pi nt - \alpha - \psi)/\sqrt{(1 + 4\pi^2k^2n^2)(1 + 4\pi^2\lambda^2n^2)} \qquad (37)$$

where $\tan \psi = 2\pi n\lambda$.

In this we have neglected another exponential term, analogous to that discussed above: we are interested only in the steady condition of excitation, once to each cycle, and not in what happens in the first cycle alone.

The effect of this other exponential term also was observed experimentally by Coppée. It is greatest at low frequency and when the phase at which the current is made is about $\pi/2$. It is obvious physically that with a slowly increasing wave stimulation will be most effective if accommodation is avoided by " making " the current near the crest of the wave.

For steady threshold excitation $U = V$ and $dU/dt = dV/dt$. The latter, on differentiating and simplifying, gives $\tan (2\pi nt - \alpha) = 2\pi n\lambda = \tan \psi$; so that $(2\pi nt - \alpha) = r\pi + \psi$, where $r$ is some integer. Hence $(2\pi nt - \alpha - \psi) = r\pi$ and $\sin (2\pi nt - \alpha - \psi) = 0$. Hence in equation (37), $U = U_0$, an interesting and unexpected result, viz., that threshold excitation occurs at the moment when U is passing through its normal " unaccommodated " value.

TABLE VIII

| $n$, cycles/sec.... | 5 | 10 | 20 | 40 | 75 | 150 |
|---|---|---|---|---|---|---|
| $t_1/\pi$ .......... | 0·102 | 0·188 | 0·306 | 0·420 | 0·517 | 0·606 |
| $n$, cycles/sec.... | 300 | 600 | 1000 | 2000 | 4000 | 10,000 |
| $t_1/\pi$ .......... | 0·723 | 0·836 | 0·897 | 0·948 | 0·973 | 0·990 |

The moment at which excitation occurs is given by $t_1 = (\alpha + \psi)/2\pi n$. If $n$ is small, excitation takes place early in the wave: if $n$ is large, excitation takes place late. Table VIII shows the time, as a fraction of the half-cycle, at which excitation occurs, for the typical case of a frog's nerve at 15° C, $k = 0{\cdot}5$ m sec, $\lambda = 10$ m sec (rather rapid " accommodation ").

Applying now the other condition for threshold excitation, viz., $V = U$, with $U = U_0$ and $\tan(2\pi nt - \alpha) = \tan \psi$ as shown already, we find,

$$I/I_0 = \sqrt{(1 + 4\pi^2k^2n^2)(1 + 1/4\pi^2\lambda^2n^2)}. \tag{38}$$

This is the general equation for the relation between intensity and frequency for threshold A.C. stimulation: it takes account of both factors, $k$ and $\lambda$. In fig. 12, $I/I_0$ is plotted, as a function of $n$, from equation (38), for two cases, one of rapid and one of rather slow "accommodation," for a normal frog's nerve at 15° C. The curves are identical above about 200 cycles/sec.

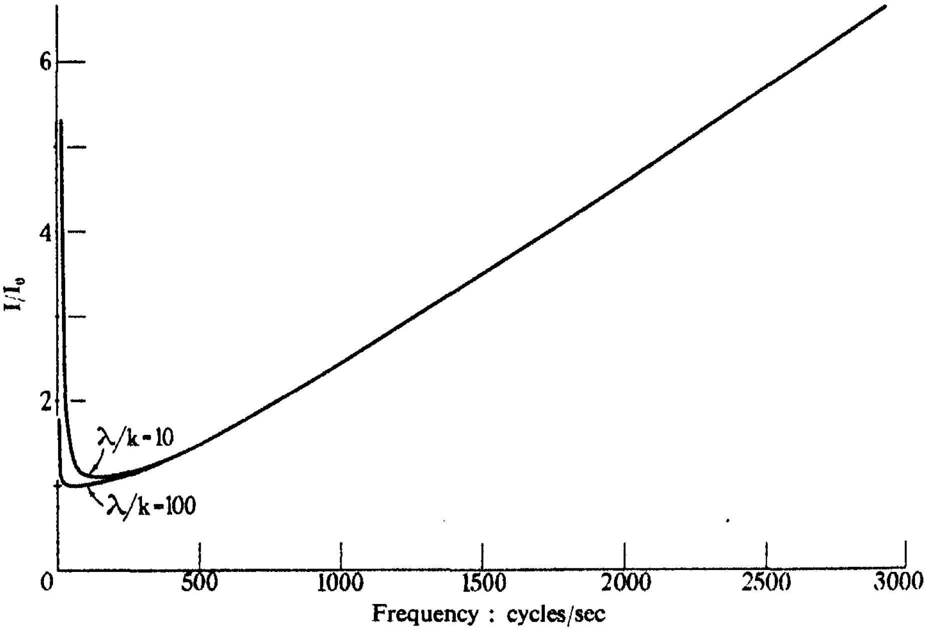


FIG. 12—Alternating current stimulation. Relation between threshold intensity and frequency, calculated from equation (38), for two cases of a frog's nerve at 15° C: (*a*) $k = 0 \cdot 356$ m sec: $\lambda = 3 \cdot 56$ m sec (very rapid "accommodation"); (*b*) $k = 0 \cdot 356$ m sec: $\lambda = 35 \cdot 6$ m sec (rather slow "accommodation").

The relation has several important characteristics:—

(i) There is an optimum frequency $n_0$ at which excitation occurs with minimum current: $n_0^2 = 1/4\pi^2k\lambda$: at this frequency $I/I_0 = (1 + k/\lambda)$.

(ii) At low frequencies $I/I_0$ becomes large: excitation becomes difficult owing to "accommodation" occurring due to the slowness of the rise and fall of the current.

(iii) At high frequencies $I/I_0$ becomes equal to $2\pi nk$, not, as Nernst's theory (1908) suggested, to its square root.

(iv) If $I/I_0$ is plotted against log $n$ the curve becomes symmetrical about a vertical axis through the optimum frequency. This has been observed experimentally by Coppée (1934) to be so.

It is remarkable that von Kries (1884, *a*), more than 50 years ago, established the existence of an optimum frequency. He stated that in frog's nerve at room temperature it was about 100 per second, while at 37° C it was more than 200, at 3–4° C less than 50 per second. He clearly recognized that the existence of an optimum frequency depends on the same factor as the rise of threshold associated with a slowly increasing current. By modern methods an optimum frequency was found by Lullies (1929, 1930), by Achelis (1930), by Renquist and Koch (1930), and by Coppée (1934). It has been fully confirmed in the present investigation, employing non-polarizable electrodes (calomel half-cells) and special arrangements for giving a pure sine-wave form to the lower, as well as to the higher, frequencies. The optimum frequency $n_0$, calculated from the formula $n_0^2 = 1/4\pi^2 k\lambda$, gives values approximately the same as observed experimentally. For example, with $k = 0\cdot5$ m sec and $\lambda = 20$ m sec, a normal case for a frog's nerve at 15° C, $n_0 = 50$: Lullies found 68 at 18–20° C: Achellis found 55 at 15° C. The matter will be discussed in detail in a later paper. Many references to the subject of alternating current stimulation are given by Coppée (1934).

The phase-differences between the current, the local potential V and the threshold U are interesting. V lags behind the current by a phase angle $\tan^{-1}(2\pi nk)$. When $n$ is small this is small: when $n$ is large it becomes $\pi/2$. U lags behind V by an angle $\tan^{-1}(2\pi n\lambda)$: this also is small when $n$ is small, and becomes $\pi/2$ when $n$ is large. Since $\lambda$ is much greater than $k$, U lags behind V more than V behind the current. The system has obvious electrical analogies.

## XIV—REPETITIVE STIMULATION

Suppose that a nerve is stimulated at regular intervals T, by one-way sub-threshold condenser discharges of time-constant RC. After an interval of about $5\lambda$ from the start, a steady condition is set up in which V and U are responding to each discharge, not from their original values $V_0$ and $U_0$, but from displaced values $V_1$ and $U_1$. We can calculate the residual effect of steady stimulation of this type on V and U, *i.e.*, the values of $V_1$ and $U_1$, at the end of any one of the intervals T, just before the next shock is applied.

We obtain an infinite series which, however, is easily summed (*cf.* Bugnard and Hill, 1935, *a*, p. 396). From equation (30), at the end of one of the intervals,

$$\begin{aligned} V_1 - V_0 &= \frac{bkE/R}{1 - k/RC}\,[e^{-T/RC} + e^{-2T/RC} + e^{-3T/RC} + \ldots \\ &\qquad - e^{-T/k} - e^{-2T/k} - e^{-3T/k} - \ldots] \\ &= \frac{bkE/R}{1 - k/RC}\left[\frac{e^{-T/RC}}{1 - e^{-T/RC}} - \frac{e^{-T/k}}{1 - e^{-T/k}}\right]. \end{aligned} \tag{39}$$

From equation (33) similarly,

$$U_1 - U_0 = RCbkE/R\left[\frac{k}{(RC - k)(\lambda - k)}\,\frac{e^{-T/k}}{1 - e^{-T/k}} + 2 \text{ similar terms}\right], \tag{40}$$

and from equation (34),

$$U_1 - V_1 = (U_0 - V_0)\left[1 + RC\lambda E/E_0\left\{\frac{e^{-T/k}}{(1 - e^{-T/k})(RC - k)(\lambda - k)} + 2\,\begin{matrix}\text{similar}\\ \text{terms}\end{matrix}\right\}\right]. \tag{41}$$

Consider equation (41) for a typical case, viz., the stimulation of a normal frog's nerve at 15° C, with $k = 0{\cdot}5$ m sec, $\lambda = 50$ m sec (average " accommodation "). Let us apply 1000 one-way condenser discharges per second, each with $RC = 0{\cdot}1$ m sec. Here $T = 1$ m sec. This is the kind of case dealt with by Bugnard and Hill (1935, *a*) and by Cattell and Gerard (1935) in their work on high-frequency excitation by condenser discharges. Make E only just below threshold, so that $E/E_0$, calculated from equation (32), is $7{\cdot}5$. Then, with these numbers equation (41) gives:—

$$(U_1 - V_1)/(U_0 - V_0) = 1{\cdot}46.$$

Thus the threshold has been raised 46% by just sub-threshold rapid condenser discharges at 1000/sec. " Accommodation," therefore, is one of the causes of the failure of nerve to respond to high-frequency one-way repetitive stimulation. Even if the earlier shocks were just effective, a state of " accommodation " would rapidly set in, in which failure would result. A stronger stimulus would then be required: this would increase the " accommodation " and a still stronger stimulus would be needed. In the case considered, increasing E from threshold to 1·85 times the threshold value for a single shock would make $(U_1 - V_1)/(U_0 - V_0) = 1 + 1{\cdot}85 \times 0{\cdot}46 = 1{\cdot}85$, so that the steady threshold at 1000/sec would be 1·85 times that for a single discharge.

With two-way shocks (*i.e.*, alternating in direction) the residual effect of steady stimulation is small, and actually of opposite sign from that just discussed; it would slightly increase the excitability, not largely diminish it. It was found by Bugnard and Hill (1935, *a*, p. 398) that two-way stimulation of high frequency is less effective than one-way in diminishing the response; and it is possible that the diminution with two-way discharges is really due, as Katz (1936) suggests, to an electrotonic block by alternate shocks outrunning and cutting off the impulses actually started. With one-way discharges of high frequency, however, the " accommodation " described above could scarcely fail to appear and considerably to raise the threshold.

## XV—DISCUSSION

The statement given above of the two time-factors in electric excitation is the simplest possible one, and it is realized only too clearly that in certain respects it is inadequate. Its chief weakness is that it deals only with "normal accommodation," in which, after full " accommodation " to a constant current, (U − V) attains its original value $(U_0 - V_0)$. It takes no account, therefore, of electrotonic changes of excitability along a nerve in which current is flowing: those are regarded as secondary, for conditions are known in which they do not occur. This weakness will often make it impossible in experimental work to obtain exact quantitative agreement with the formulae given above, for electrotonic changes of excitability will have set in during the passage of the current considered. Moreover, no attempt has been made to deal with possible alterations of V and U when actual excitation occurs: we have considered merely the processes leading to excitation. The refractory period, absolute and relative, and its alterations with continued stimulation; the supernormal phase; repetitive response to a strong long-lasting current; the nature of the action potential; these and other such matters have been omitted from the present discussion.

It might be regarded as a further weakness that no physical model has been proposed. The surface of the nerve fibre has not been supposed to act as an electrical condenser; the " local potential " has not been identified with the electrotonic potential; the concentration of ions at a semi-permeable membrane has not been assumed to determine excitation; the constituents of a sensitive surface have not been imagined to flow, or to alter their shape, under the influence of a current, and so to lead to " accommodation." The " local potential " is no more than

Lucas's "excitatory disturbance": it is altered by passing a current through the nerve: we do not know what it is, so we call it V. We assume that V tends to revert to its original value $V_0$, in a simple exponential manner with time-constant *k*, when the current is removed. This is justified by the result. We assume that when the current is reversed the consequent change of V is reversed too: this necessarily happens if the change of V is proportional to the current; it would not happen if the change of V were proportional to the energy, *i.e.*, to the square of the current.

All the facts of excitation lead to the idea of a "threshold," of a level at which a discontinuity of some kind sets in. We suppose that excitation occurs when V reaches a certain level U: that for very short currents this level U is constant: that for longer currents it rises: that its rate of rise at any moment is determined by the value of V at that moment: and that when V is restored to its original value, U returns to its original level also in a simple exponential manner with time-constant $\lambda$. We have made no assumption of the physical nature either of U or of $\lambda$: but those we have made as to their behaviour express the facts simply, and further hypotheses are at the moment unnecessary.

Although not based on any particular physical theory the behaviour of U and V is easy to appreciate physically. A simple hydraulic model, however, fig. 13, may help. Two tanks A and B are filled with a viscous fluid. They are joined by two pipes, one with a tap K, which controls the flow of liquid from A to B, the other with a pump P. Tank B contains a float C connected to a piston E running in a cylinder F. The tap L controls the rate of flow of liquid from F to B and vice versa.

Let us take the level of the fluid in B as V and assume that when this level reaches a hole, or a contact, D in the float C "excitation" occurs. The height of D represents the "threshold" U. With the pump stationary and taps K and L open, the tanks are in equilibrium, and the level V of the fluid is $V_0$, that U of the hole D is $U_0$. Starting the pump is equivalent to "making" a current through the nerve.

This model would obey all the equations given in this paper. Strength-duration curves could be made with it, by pumping fluid in or out at different rates. Progressive currents, condenser discharges, gaps in constant currents, even alternating current, could be applied to it. The time constants *k* and $\lambda$ could be adjusted independently by setting the taps K and L. The "true rheobase," the "observed rheobase," the utilization time, and the constant-quantity relation at short times, could all be reproduced.

The inefficiency of "make" at the anode could be demonstrated by reversing the pump: V, starting below U and moving away from and faster than U, would never be caught by it. Excitation at "break" of constant current could be realized by continuing the reversed current until a "fully accommodated" condition had been reached, in which, with the pump going and the fluid pouring back through K, the float and the surface of the liquid were at steady levels lower than originally: then stopping the pump would cause a rapid rise of the liquid, a slower rise of the float, and

V might catch U as in figs. 2 and 4. All the ordinary phenomena of excitation discussed above could thus be shown in the model.

The inadequacy of a single time-constant to account for all the facts in electric excitation has long been realized. Fabre's " *constante linéaire* " (1927, etc.), which he finally proved (1931) to be independent of the " chronaxie," represents the first direct introduction of what we have here called $\lambda$, the time-constant of accommodation, *see* § X. Schriever's (1930, 1932, *a*, *b*) " *Einschleichzeit*," which he called the " chronherpie," and derived from exponentially increasing currents, is $\lambda/2{\cdot}89$, just as the " chronaxie " is $k/1{\cdot}44$. Schriever's experiments suggested that " *Einschleichzeit* " and " chronaxie " are somehow related, which Solandt's experiments show they are not: $\lambda$ can be caused to vary, over a wide range, by changes of ionic concentration, without significant alterations of $k$. Blair's theory (1932, 1935) involves only one time-constant, $k$, but it requires a variable threshold, capable of altering instantaneously in accordance with the applied current: it is referred to below. The most complete, and largely successful, attempt to deal with

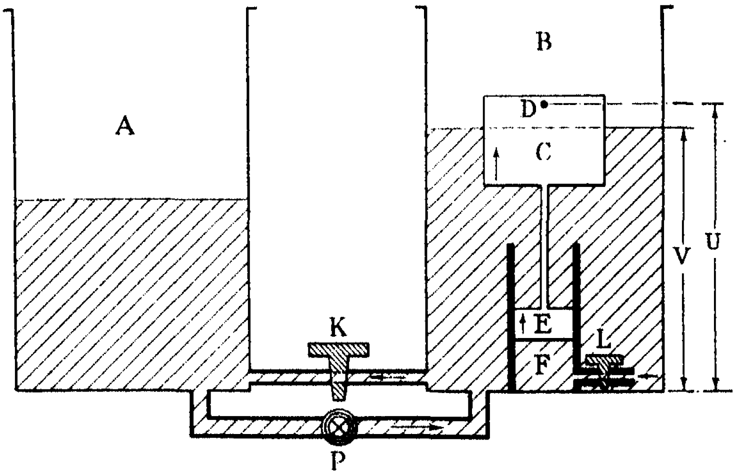


FIG. 13—Hydraulic model to illustrate excitation and " accommodation." P = pump, producing current I from tank A to tank B or vice versa. A difference of level between the two tanks tends to disappear with time-constant $k$ depending on the opening of the tap K. The height of the liquid in B, *i.e.*, of the " excitatory disturbance," is V. A float C rises, or falls, in tank B, under the control of a piston E in the cylinder F. The rate of rise of C (" accommodation ") depends upon V and upon the viscous resistance in tap L: the float tends to adjust itself to any given level V with a time-constant $\lambda$ depending upon the opening of L. Excitation is supposed to occur when the level of the liquid reaches the hole (or contact) D in the float. The height of D is U, the " threshold."

the facts of which the older theories gave no adequate account is that of Monnier.

Monnier's scheme (1934, *a*, *b*) of electric excitation is based upon the hypothesis of a " state of excitation " which is assumed to wax and wane, following a characteristic course, when a constant current is passed continually through a nerve. This " state of excitation " is exemplified in two models, (i) an electrical one (1934, *a*, p. 14) involving two separate condensers and therefore two time-constants, (ii) a hydraulic one (p. 20) involving two separate resistances to fluid flow (*cf.* fig. 13 above) and hence again two time-constants. The " state of excitation," of intensity proportional to the current, and starting from zero at the moment the current is " made," is assumed to rise to a maximum and then to fall gradually to its original value, at which it remains so long as the current is continued. If the maximum is above a certain fixed " threshold " level, excitation results. On breaking the current after the steady condition has been reached, the " state of excitation " passes through a similar course in the opposite sense. If $v$ measures the " state of excitation " and $i$ the current, Monnier assumes that,

$$v = ri\,(e^{-t/\tau_2} - e^{-t/\tau_1}).$$

Here $r$ is a constant, $\tau_1$ and $\tau_2$ are times.

No single physical factor is known in nerve to wax and wane in this manner during the passage of a constant current. If excitation is in any way determined by the rise of electrotonic potential, at or near the electrode, as recent work by Harris and Rosenberg (1935, 1936) and by Schmitz and Schaefer (1933, 1934) strongly suggests, we are still further from any single physical agency corresponding to Monnier's " state of excitation "; for the electrotonic potential remains at least approximately constant, after its initial rise, during the continued passage of a current through a nerve. If, however, we regard Monnier's " state of excitation " at any moment as simply measuring the amount by which the strength of a very short shock required for excitation at that moment is less than its original value, then the success of Monnier's theory in accounting for the facts is explained. In equations (11), (16), (24''), (29), and (34) above, $(U_0 - V_0)$ represents the minimal strength of a very short shock required in the resting nerve for excitation, $(U - V)$ represents the minimal strength at any moment during the passage of a current. Thus Monnier's " state of excitation " is simply $(U_0 - V_0) - (U - V)$, and we see from equation (16), for constant current, that

$$(U_0 - V_0) - (U - V) = \frac{bkI}{1 - k/\lambda}\,(e^{-t/\lambda} - e^{-t/k}).$$

This is exactly of the assumed form. Thus Monnier's $\tau_2$ is $\lambda$, his $\tau_1$ is $k$, and his " state of excitation " is proportional to the current i which produced it.

For constant currents the value of $(U_0 - V_0) - (U - V)$ is simply the difference of two exponentials: with other forms of current it is slightly more complicated. For example, for a pulse of very short duration, it is $bQ\,[e^{-t/k}/(1 - k/\lambda) - e^{-t/\lambda}/(\lambda/k - 1)]$. For a constant current pulse, four exponentials are involved (equation (16′)). For a linear progressive current it is $I_0\,\mu\lambda bk\,[1 - \lambda e^{-t/\lambda}/(\lambda - k) + ke^{-t/k}/(\lambda - k)]$. For an exponential current it is $I_2\,bk/(1 - a/\lambda)\,[e^{-t/\lambda}/(1 - k/\lambda) - e^{-t/a}/(1 - k/a)]$. The simple difference is a special case for constant current.

The theory proposed in this paper, therefore, has as one of its consequences the existence of a function $(U_0 - V_0) - (U - V)$ which has the same properties as Monnier attributes to his " state of excitation." It is natural, therefore, that Monnier's theory should account for the facts of excitation, by various kinds of current, to the same extent as the present one. The latter is more explicit in that it deals with two separate factors, " local potential " or " excitatory disturbance," on the one hand, and " threshold " on the other, and attributes the two time-factors to these respectively; and it shows why the " state of excitation," for the particular case of a constant current, should behave as Monnier supposes. Monnier's theory has the same limitations as the present one.

H. A. Blair (1935, *b*), extending the theory proposed in his previous papers (1932, *a*, *b*, 1934), has attempted to relate excitation by linearly rising currents to excitation by constant currents, using only a single time-constant *k* (the reciprocal of the one used here). He introduces, however, the subsidiary hypothesis that excitation occurs, not when the excitatory process reaches a given fixed level, but one which depends on the value of the current *at the moment* when that process becomes adequate. It is physically very unlikely that the degree of " accommodation " (*i.e.*, the total change of threshold) at any moment should depend only on the current *at* that moment; " accommodation " is a gradual process, with a time-constant of its own, and the total change of threshold is bound to depend upon the value of the current at all moments up to that at which excitation occurs. Otherwise, as Rushton (1934) points out, a number of surprising conclusions can be drawn, which are contrary to observed fact. Not unnaturally, therefore, Blair's equations fail at the longer times: they would fail for many of the effects discussed above, particularly those referring to the changes of excitability produced by a sub-threshold stimulus. His theory will be referred to again in a paper

(Hill, 1936), dealing more in detail with the strength-duration curve. It does not agree with the constant quantity relation at very short times.

I am indebted to many colleagues, particularly to Drs. D. Y. Solandt, H. Rosenberg, B. Katz, and W. A. H. Rushton, for their help, and for calling attention to various aspects of the problem. My own interest in electric excitation was aroused by Keith Lucas, under whose guidance a first (and a rather naive) contribution to it was made twenty-five years ago. The theory of " accommodation " then proposed was attached to an unnecessarily complicated theory of excitation, and it neglected the essential fact that the system tends to revert of itself to its initial condition when the cause of "accommodation" is removed; it contained, nevertheless, the germ of the present theory. Lucas's inspiration was the basis of that early attempt, as his experimental work is still largely that of the present one. It will be evident how much this treatment of the problem of electric excitation owes to the work of others, from von Kries onwards, but particularly to Keith Lucas's colleague and frequent correspondent, Professor Louis Lapicque, and his School.

## SUMMARY

There are two time-factors in electric excitation, that ($k$) of the " excitatory disturbance " or " local potential " V, and that ($\lambda$) of " accommodation " or change of " threshold " U. $\lambda$ is much greater than $k$ and independent of it.

From the constant-quantity relation for excitation by currents of short duration it is concluded that, under an instantaneous discharge, the " local potential " V is raised instantly by an amount proportional to the discharge. After the discharge, V reverts exponentially to its initial value $V_0$ with time-constant $k$.

It is possible, by integration, to calculate $(V - V_0)$ for any form of applied current. Neglecting "accommodation," the ratio of the threshold *quantity* for short times to the threshold *current* for long times is $k$. The " excitation time " (Lucas), or the " chronaxie " (Lapicque) is $k \times \log_e 2 = 0{\cdot}693k$.

For short discharges, excitation occurs when V becomes equal to $U_0$, the resting " threshold." For longer discharges, however, the " threshold " U alters at a rate depending (*a*) at any moment, on the value of V at that moment, and (*b*) upon its natural tendency to revert exponentially to its initial value with time-constant $\lambda$.

$k$ is the time-constant of the " rate at which the excitatory disturbance

subsides"; λ is that of the rate at which, after "accommodation," the "threshold" reverts to its initial level.

It is possible, by integration, to calculate $(U - U_0)$ for any form of applied current.

It is supposed, in general, that excitation occurs when V becomes equal to U. Assuming that the changes of V and U are similar, but in opposite directions, at anode and cathode, it is shown that "excitation at break" is a necessary consequence of "accommodation" and requires no special theory.

It is possible to calculate—

(*a*) the form of the strength-duration curve (constant current pulses, or condenser discharges);

(*b*) the conditions for excitation at break, or at gap in constant current;

(*c*) the "utilization time" for currents of any form;

(*d*) the effects of "accommodation" on the "rheobase" and "chronaxie": with rapid "accommodation" both are considerable;

(*e*) the relation between final intensity and time of rise, with linearly increasing currents, and the slope of the "minimal current gradient";

(*f*) the relation between final intensity and time-constant of rise, with exponentially increasing currents;

(*g*) the relation between strength and frequency with alternating current, and the existence and position of the optimum frequency;

(*h*) the changes of excitability during and after the passage of sub-threshold currents of any form;

(*i*) the lowered excitability during sub-threshold high-frequency one-way stimulation.

These calculations can be made with observed quantities and in absolute units.

Several methods of determining experimentally the value of λ, the time-constant of "accommodation," are discussed. They lead to consistent results.

λ is considerably affected by temperature, and largely affected by the Ca-ion concentration. The influence of Ca on "utilization time," on "summation interval," and on "minimal current gradient" is due to its effect on λ.

Fabre's "*constante linéaire*," Schriever's "*Einschleichzeit*" (multiplied by 2·89), and Monnier's $\tau_2$, are shown to be the same thing as $\lambda$. Monnier's "*état d'excitation*" is shown to be $(U_0 - V_0) - (U - V)$.

A hydraulic model is described which, with two independent time-constants, obeys the relations here deduced for the excitation of nerve, and allows the changes of V and U to be visualized.

The limitations of the theory are discussed. No attempt is made to account for electrotonic changes of excitability. Conditions are known in which these do not occur, or are reversed, so they must be regarded as secondary; usually, however, they will complicate (but not disguise) the relations predicted.

No specific physical or chemical theory is offered of the nature of "local potential" V, of "threshold" U, or of their time-constants $k$ and $\lambda$. Their behaviour only is discussed. They are of a type, however, which could readily be expressed in physical or chemical terms.

## REFERENCES

Achelis, J. D. (1930). 'Pflügers Arch.,' vol. **224**, p. 217.

Bishop, G. H. (1928). 'Amer. J. Physiol.,' vol. **84**, p. 417.

Blair, H. A. (1932, *a*). 'J. gen. Physiol.,' vol. **15**, pp. 709, 731.

—— (1932, *b*). *Ibid.*, vol. **16**, pp. 165, 177.

—— (1934). *Ibid.*, vol. **18**, p. 755.

—— (1935, *a*). 'J. cell. comp. Physiol.,' vol. **6**, p. 291.

—— (1935, *b*). 'Amer. J. Physiol.,' vol. **111**, p. 515.

Blumenfeld, E. (1925). 'Biochem. Z.,' vol. **156**, p. 236.

Bonnardel, R. (1934). Thesis: "Contribution expérimentale à l'étude de l'excitabilité," Paris, Imprimerie Chantenay.

Bouman, H. D. (1936). (*In preparation.*)

Buchloh, W. (1931). 'Ber. ges. Physiol.,' vol. **61**, p. 343.

Bugnard, L., and Hill, A. V. (1935, *a*). 'J. Physiol.,' vol. **88**, pp. 383, 394, 416.

—— (1935, *b*). *Ibid.*, vol. **88**, p. 425.

Cardot, H., and Laugier, H. (1912, *a*). 'C.R. Soc. Biol., Paris,' vol. **72**, p. 230.

—— (1912, *b*). 'J. Physiol. Path. gen.,' vol. **14**, p. 263.

—— (1913). *Ibid.*, vol. **15**, p. 1134.

Cattell, McK., and Gerard, R. W. (1935). 'J. Physiol.,' vol. **83**, p. 407.

Chweitzer, A. (1935). 'C.R. Soc. Biol. Paris,' vol. **120**, p. 626.

Cluzet, J. (1908). 'J. Physiol. Path. gen.,' vol. **10**, p. 392.

Colle, J. (1933, *a*). 'Arch. int. Physiol.,' vol. **37**, p. 410.

—— (1933, *b*). 'C.R. Soc. Biol., Paris,' vol. **115**, p. 207.

Coppée, G. (1934). 'Arch. int. Physiol.,' vol. **38**, pp. 239, 251; vol. **40**, p. 1.

Delville, P. (1934). *Ibid.*, vol. **40**, p. 83.

Eichler, W. (1932, 1933). 'Z. Biol.,' vol. **92**, p. 331; vol. **94**, p. 177.

—— (1933). *Ibid.*, vol. **93**, p. 527.

Erlanger, J., and Blair, E. A. (1931, *a*). 'Amer. J. Physiol.,' vol. **99**, p. 108.

Erlanger, J., and Blair, E. A. (1931, *b*). *Ibid.*, vol. **99**, p. 129.

Fabre, P. (1927, *a*). ' C.R. Acad. Sci., Paris,' vol. **184**, pp. 699, 1486.

—— (1927, *b*). *Ibid.*, vol. **185**, pp. 300, 1216.

—— (1928). *Ibid.*, vol. **186**, p. 1150.

—— (1931). ' C.R. Soc. Biol., Paris,' vol. **108**, pp. 1248, 1250.

—— (1933, *a*). ' C.R. Acad. Sci., Paris,' vol. **196**, p. 134.

—— (1933, *b*). ' J. Physiol. Path. gen.,' vol. **31**, pp. 313, 766, 781.

—— (1934, *a*). ' C.R. Soc. Biol., Paris,' vol. **116**, p. 1063.

—— (1934, *b*). *Ibid.*, vol. **116**, p. 1065.

—— (1934, *c*). *Ibid.*, vol. **117**, p. 31.

Fabre, P., Quesnoy, P., and Berteaux, J. (1934). *Ibid.*, vol. **115**, p. 1431; vol. **116**, p. 179.

Fabre, P., and Swyngedauw, J. (1933). *Ibid.*, vol. **113**, p. 765.

Fick, A. (1864). *See* Fick's ' Gesammelte Schriften,' vol. **3**, p. 133.

Gildemeister, M. (1904). ' Pflügers Arch.,' vol. **101**, p. 203.

—— (1908). *Ibid.*, vol. **124**, p. 447.

—— (1913). ' Z. Biol.,' vol. **62**, p. 358.

—— (1929). ' Ber. Sächs. Akad. Wiss.,' vol. **81**, p. 303.

Harris, D. T., and Rosenberg, H. (1935). ' J. Physiol.,' vol. **84**, p. 11P.

—— (1936). *Ibid.* (*in the press*).

Hill, A. V. (1910). *Ibid.*, vol. **40**, p. 190.

—— (1935, *a*). *Ibid.*, vol. **83**, p. 30P.

—— (1935, *b*). " Advances in Modern Biology," Moscow, vol. **4**, p. 131.

—— (1936). ' Proc. Roy. Soc.,' B (*in the press*).

——, Katz, B., and Solandt (1936). (*In preparation.*)

Kahn, E. (1911). ' Pflügers Arch.,' vol. **143**, p. 428.

Katz, B. (1936). ' J. Physiol ' (*in the press*).

von Kries (1884, *a*). ' Ber. naturf. Ges. Freiburg, i.B.,' vol. **8**, p. 170.

—— (1884, *b*). *Ibid.*, p. 265.

—— (1884, *c*). ' Arch. Anat. Physiol., Lpz.,' p. 337.

Lapicque, L. (1908, *a*). ' C.R. Soc. Biol., Paris,' vol. **64**, pp. 6, 336.

—— (1908, *b*). ' J. Physiol. Path. gén.,' vol. **10**, p. 601.

—— (1926). " L'excitabilité en fonction du temps," Presses Universitaires, Paris

Laugier, H. (1921). ' Thesis,' Paris, Imprimerie Paul Marmy.

Liberson, W. (1934). ' C.R. Soc. Biol., Paris,' vol. **116**, p. 1319.

Lucas, K. (1907, *a*). ' J. Physiol.,' vol. **35**, p. 310.

—— (1907, *b*). *Ibid.*, vol. **36**, p. 253.

—— (1908). *Ibid.*, vol. **37**, p. 459.

—— (1910). *Ibid.*, vol. **39**, p. 461.

Lullies, H. (1929). Quoted from Junk's ' Tabulae Biologicae Periodicae ' (1931), vol. **1** (= Tab. Biol. 7).

—— (1930). ' Pflügers Arch.,' vol. **225**, p. 98.

Mines, G. R. (1908). ' J. Physiol.,' vol. **37**, p. 408.

Monnier, A. -M. (1934, *a*). " L'excitation électrique des tissus," Hermann et Cie., Paris.

—— (1934, *b*). ' Bull. Soc. Philomathique, Paris,' vol. **117**, p. 107.

Nernst, W. (1908). ' Pflügers Arch.,' vol. **122**, p. 275.

Renquist, Y., and Koch, H. (1930). ' Skand. Arch.,' vol. **59**, p. 279.

Rosenberg, H. (1935). ' J. Physiol.,' vol. **84**, p. 50.
Rushton, W. A. H. (1931). *Ibid.*, vol. **72**, p. 265.
—— (1932). *Ibid.*, vol. **75**, p. 16P.
—— (1934). ' J. gen. Physiol.,' vol. **17**, p. 481.
Schaefer, H., and Schmitz, W. (1934). ' Pflügers Arch.,' vol. **233**, p. 700.
Schmitz, W., and Schaefer, H. (1933). *Ibid.*, vol. **232**, p. 782; vol. **233**, p. 229.
Schriever, H. (1930). ' Z. Biol.,' vol. **91**, p. 173.
—— (1932, *a*). *Ibid.*, vol. **93**, p. 123.
—— (1932, *b*). *Ibid.*, vol. **93**, p. 249.
Scott, D. (1934). ' J. Physiol.,' vol. **82**, p. 321.
Solandt, D. Y. (1935). " Advances in Modern Biology," Moscow, vol. **4**, p. 144.
—— (1936). (*In preparation.*)
Suzuki, M. (1932). ' Pflügers Arch.,' vol. **230**, p. 263.
Wyss, O. A. M. (1934). *Ibid.*, vol. **233**, p. 754.

---

612 . 816

# The Measurement of " Accommodation " in Nerve

By D. Y. Solandt, University of Toronto Fellow

(From the Department of Physiology, University College, London)

(*Communicated by Professor A. V. Hill, Sec.R.S.—Received December* 10, 1935)

When an electric current, insufficiently strong to excite, is removed from a nerve the " excitatory disturbance " (Lucas, 1910) or " local potential " (Hill, 1936) reverts to its resting value. The time-constant of this decay, assumed exponential, is the time-constant of excitation, $k$. If this assumption is correct, the chronaxie is $0 \cdot 693k$.

When an electric current passing through a nerve lasts for a very short time the critical value of the " local potential " required for excitation. that is the threshold, is constant. The previous history of the nerve is not involved and the only time-constant is $k$. If the current lasts for a longer time, as with constant currents near the rheobase, with linearly or exponentially increasing currents, or with low frequency alternating currents, the threshold rises: the threshold for a slowly increasing current is well-known to be higher than for a rapidly increasing one. This change in threshold is called " accommodation " (Nernst, 1908). If the rise in threshold occurs because of the rise in " local potential," the threshold will revert to its resting value when the " local potential " reverts to its resting value. The time-constant of the return, assumed exponential, of the threshold to its resting level is λ, the time-constant of "accommodation." If this assumption is correct, the time to half return is 0·693 λ.

In defining $k$ and λ the return to the resting condition, not the rise from the resting condition, is used. The decay is a true property of the tissue independent of the particular form of the applied current.

The experiments described in this paper were designed to measure the time-constant of the "accommodation" process, through excitation by exponentially increasing currents. Hill (1935 and 1936) has outlined the theoretical basis of this method of obtaining λ. If the exciting current, instead of reaching the threshold value instantly, rises exponentially, then the threshold exponential current (I) divided by the rheobasic constant current ($I_0$) should bear a nearly linear relation to the time-constant of current rise ($a$). This is true only when these time-constants ($a$ and λ) are large compared with the time-constant of excitation ($k$). In practice $I_0$ is first observed and then values of I measured for various values of $a$, care being taken to keep $a$ large compared with $k$. If $I/I_0$ is plotted against $a$, the theory predicts a straight line, the slope of which is $1/\lambda$. The validity of the theory is thus simply tested. If a straight line is obtained, over the predicted range of values of $a$, the theory is thereby borne out and this experimental procedure provides a convenient method of measuring λ.

## METHOD

Exponentially increasing currents were produced through the use of a variable condenser with appropriate resistances (Lapicque, 1908 and 1926, p. 135), as shown in fig. 1. The effective resistance shunting the condenser is the harmonic mean of the resistances of the two arms of the circuit. The only significant resistance in the battery arm of the circuit is $R_1$. The resistance of the preparation arm is $R_2$ plus the preparation resistance $R_p$. Thus the effective resistance is

$$R = \frac{R_1(R_2 + R_p)}{R_1 + R_2 + R_p}.$$

If R is expressed in megohms and the capacity of the condenser C in microfarads then

$$RC = T,$$

where T is the time-constant of current rise (time taken for the current to reach $(1 - 1/e)$ of its final value) expressed in seconds.

In practice the threshold is found for a current of instantaneous rise (no condenser in the circuit), this being the observed rheobase. Thresholds are then measured for currents of greater and greater time-constant of rise. A return series is performed and the average threshold obtained for each time-constant of current rise used. These average values are divided by the observed rheobase and plotted as ordinates against the corresponding time-constants of current rise as abscissae. The slope of this line gives the reciprocal of $\lambda$ expressed in the same units as the time-constant of current rise. Thresholds are actually measured in volts at the potential source, as this is simpler than measuring the small currents which produce excitation. The current is, of course, proportional to the voltage and, as all thresholds are referred to a rheobase, the absolute values are of no importance.

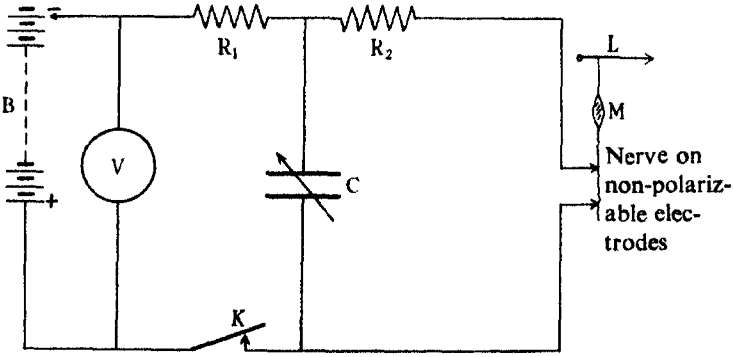


FIG. 1—Circuit used for measuring "accommodation" in nerves of frogs, fishes, and crabs. Potentiometer used for fine adjustment of voltage not shown. V = voltmeter; C = variable condenser up to 1·0 μF; L = lever; M = muscle; K = key.

In all experiments minimal muscle twitch was taken as index of nerve excitation.

## EXPERIMENTS ON FROG'S NERVE

These were performed with $R_1$ and $R_2$ each equal to ½ megohm and C variable up to 1·0 μF. The preparation resistance was measured regularly and was as high as 80,000 ohms. Time-constants of current rise up to about 0·27 second could thus be obtained. The sciatic-gastrocnemius preparation was used. It was mounted in a chamber fashioned from a block of paraffin-wax which carried two calomel half-cell electrodes. These non-polarizable electrodes each dipped into a pool of frog Ringer's solution (6·75 gm NaCl, 0·20 gm $CaCl_2$, 0·15 gm KCl made to 1 litre with distilled water) in which was mounted a sheet of

Ringer-soaked wood. The free upper edges of these pieces of wood made contact with the nerve. Normally, thin sheets of wood were used and the nerve was suspended on the sharpened edges.

Thick (1 cm) wood blocks were used in certain experiments. The separation of the two points of contact with the nerve could be varied from 1 mm to 30 mm. The muscle was always connected to a lever to facilitate observation of the minimal twitch.

The polarization at the electrodes was tested by connecting a very fast critically damped galvanometer (Moll " Micro-," period 1/5 second) in series with the preparation. Pressing the key, with no condenser in the circuit, should result in the current through the preparation almost instantly assuming a fixed value. With this arrangement polarization can be detected as a creeping of the galvanometer after the first quick movement. With the high circuit resistances used and no shunt across the preparation no polarization could be detected using the calomel half-cells. Using carefully chlorided silver wires, instead of the calomel half-cells, some polarization was present. The results were quantitatively the same whether the nerve was mounted directly on the wires, or whether the wires simply dipped into the pools of Ringer's solution in place of the calomel half-cells. A low-resistance shunt placed across the preparation greatly aggravated the tendency to polarization. Even the calomel half-cells showed slight polarization under these conditions, the chlorided silver wires marked polarization. No shunt across the preparation was used in any of the experiments described in this paper.

Two methods were used to test the effects of various ions on " accommodation." One was to soak the preparation in Ringer's solution to which the desired salt had been added in the form of an isotonic solution. The other method was to inject a concentrated solution of the desired salt into the dorsal lymph sac of the frog a number of hours before dissection. In either case the wooden electrodes were soaked in a solution of approximately the same ionic composition as the environment to which the nerves had been exposed. The fluid into which these and the calomel electrodes dipped was similarly constituted.

## EXPERIMENTS ON FISH NERVE

These were performed with the same circuit constants as for frog's nerve. The fish was pithed. A segmental motor nerve was cut centrally and dissected out for over an inch. It was suspended vertically from a strip of filter paper leading from a vessel containing a calomel half-cell in Ringer's solution. With the rays the slow flow of fluid down the nerve from the filter paper served to keep the preparation in good condition for 6 or 7 hours with no further attention. The other electrode was similarly constituted, the filter paper making contact with the nerve at its entrance into the muscle.

Muscle twitch, with the muscle *in situ*, was taken as index of nerve excitation.

The elasmobranch Ringer's solution, used for the experiments on rays (" skates "), contained 22·0 gm NaCl, 0·52 gm KCl, 0·44 gm $CaCl_2$, 0·47 gm $MgCl_2$, and 29·0 gm urea, made to 1 litre with distilled water. The teleost Ringer's solution used on the flatfish (" plaice ") contained 13·5 gm NaCl, 0·60 gm KCl, 0·25 gm $CaCl_2$, and 0·35 gm $MgCl_2$, made to 1 litre with distilled water (Young, 1933).

## EXPERIMENTS ON CRAB'S AND LOBSTER'S NERVE

These experiments required different circuit constants, as " accommodation " is much slower than in the nerves of frogs or fishes. Condenser capacities up to 4 $\mu$F were used, $R_1$ and $R_2$ each were equal to 1·0 megohm. The preparation resistance was about 20,000 ohms so time-constants of current rise up to about 2·02 seconds were available. The preparations were always made from the walking legs. In some cases the nerve was dissected free from adjacent tissue, this operation requiring considerable care. The simpler method of pulling the shell and attached muscles away from the nerve after cutting the hard parts of the joint was sometimes used. As a rule both meropodite and carpopodite were thus removed, one at a time, leaving only propodite and dactylopodite.

This " pulling operation " was performed under sea-water and the nerve was found to float free in good condition. The cut end of the nerve was then tied with a fine thread and the preparation set up in a stimulating chamber as with the frog's nerve preparation. Minimal movement of the dactylopodite on the propodite, which was held firmly in the chamber, was taken as index of nerve excitation. A lever was usually attached to the dactylopodite to facilitate observations of movement. As a rule the extensor tendon to the dactylopodite was cut and only flexor response used.

The preparation and electrodes were kept moist with ordinary sea-water. The effect of various ions was tested by the addition of suitable salts, in the form of isotonic solutions, to the sea-water.

## EXPERIMENTS ON HUMAN NERVE

The circuit shown in fig. 2 was used to investigate " accommodation " in human nerve. The use of low resistances and a high capacity provided the required time-constants of current rise with the comparatively high current required for this method of excitation, without the use of high battery potentials. Different time-constants of current rise were obtained by varying one of the resistances instead of the condenser capacity. With each setting of the resistance $R_1$ (variable up to 10,000 ohms) a threshold was obtained with and without the condenser in the circuit. Each reading could thus be referred to a rheobase obtained under identical conditions. In every case the ulnar nerve was stimulated at the elbow. A copper or zinc electrode 1 cm square, covered with saline-soaked cotton gauze was placed over the nerve. A similar but larger indifferent electrode was used on the other side of the arm. The preparation resistance, with this system of electrodes, lay between 3000 and 5000 ohms and could be reduced below this point by scrubbing the skin with ether and alcohol and then soaking in normal saline. This treatment rendered the skin resistance more constant as well as reducing it. The resistance was measured, usually by applying a known voltage and measuring the resulting current, before and after each series of threshold determinations. Wheatstone Bridge measurements gave the same results.

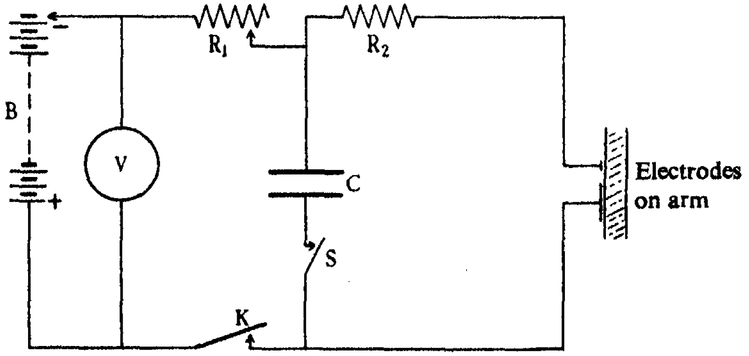


FIG. 2—Circuit used for measuring " accommodation " in human nerve. V = voltmeter; $R_1$ up to 10,000 ω; $R_2$ = 50,000 ω; C, fixed condenser (10 μF); S = switch; K = key.

Tendon twitch, detected by palpation at the wrist, was taken as index of nerve excitation.

The effect of skin capacity on the form of the current rise through the arm was tested by connecting a Downing moving-coil oscillograph in series with the preparation. Photographic records were taken using currents of various time-constants of rise. The shape of the current rise and the time-constant of the rise were checked with the preparation in the circuit and with a non-inductive resistance of the same value substituted for the preparation. These two conditions gave identical results. There was no evidence of any distortion introduced by the preparation.

## RESULTS

### “ *Accommodation* ” *in the Sciatic Nerve of the Frog*

Preparations from *Rana esculenta* (Hungarian) were used for the majority of these experiments. The experiments on normal “ accommodation ” and on the effect of Ca were also performed on preparations of *Rana temporaria* (Belgian and English) with essentially the same results. All of the experiments were made at room temperature (17–22° C) unless stated otherwise.

TABLE I—EXAMPLE OF DATA OBTAINED BY EXCITING FROG'S SCIATIC NERVE WITH EXPONENTIALLY RISING CURRENTS

Preparation from *Rana esculenta* soaked in normal frog Ringer's solution for $1\frac{1}{2}$ hours. Temperature = 20° C

| RC (m sec) | E ↓ (volts) | E ↑ (volts) | Average of E | $\frac{\text{Threshold}}{\text{Rheobase}} = \frac{E}{E_0}$ |
|---|---|---|---|---|
| 0 | 1·53 | 1·57 | 1·55 = $E_0$ | 1·00 |
| 10·8 | 2·51 | 2·59 | 2·55 | 1·65 |
| 27·0 | 4·10 | 4·28 | 4·19 | 2·70 |
| 54·0 | 6·32 | 6·58 | 6·45 | 4·16 |
| 81·0 | 8·70 | 9·00 | 8·85 | 5·70 |
| 108·0 | 10·9 | 11·7 | 11·3 | 7·28 |
| 135·0 | 13·1 | 13·9 | 13·5 | 8·70 |
| 162·0 | 15·3 | 16·1 | 15·7 | 10·1 |
| 189·0 | 17·8 | 18·6 | 18·2 | 11·7 |
| 216·0 | 20·0 | 21·2 | 20·6 | 13·3 |
| 243·0 | 22·7 | 23·7 | 23·2 | 15·0 |
| 270·0 | 25·6 | 26·2 | 25·9 | 16·7 |

“ Accommodation ” slope = 57·5
$\lambda$ = 17·4 m sec.

Table I shows a typical set of readings and from these the curve in fig. 3 has been drawn. The agreement between the two series of thresholds obtained is good. The threshold of excitation is higher when the trials are made with inadequate stimuli which are being increased in strength than when made with adequate stimuli being decreased in strength. The slope of "accommodation" is the same in each case, the only precaution necessary being to use one routine throughout. In all the experiments described inadequate stimuli were used and increased till the threshold was reached.

The averages, referred to the rheobase ($E/E_0$), as shown in Table I, when plotted against the corresponding time-constants of current rise, yield a straight line as shown in fig. 3. The slope of this line (called the "accommodation" slope) is 57·5, the reciprocal being 17·4 m sec, which is λ, the time-constant of "accommodation" for this preparation. Table II includes results calculated in this way from experiments on preparations from a number of normal Winter (November–April) frogs.

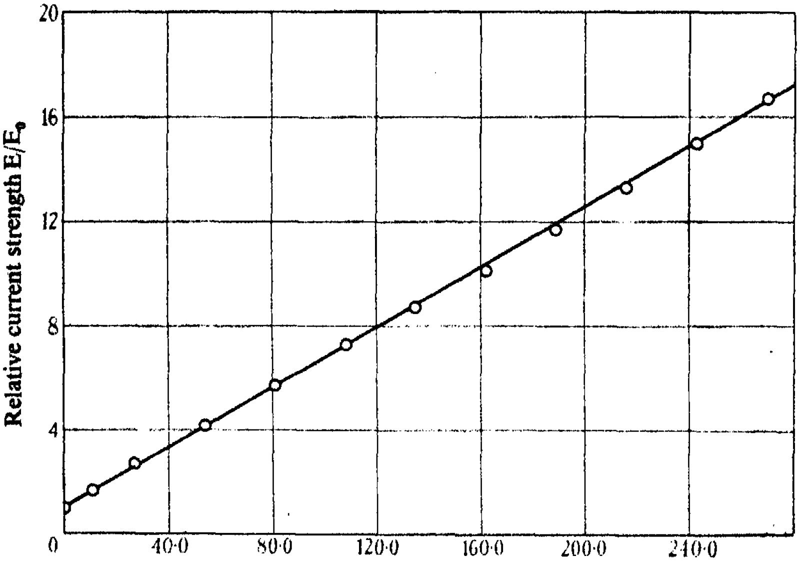


FIG. 3—"Accommodation" curve. Sciatic nerve of *Rana esculenta*. Drawn from data in Table I.

As Hill (1936) has pointed out the observed rheobase ($I_1$) is slightly higher than the actual rheobase ($I_0$). He gives the following relation—

$$\frac{I_1}{I_0} = \left(\frac{\lambda}{k}\right)^{\frac{1}{\lambda/k-1}}.$$

By substituting the values of λ and $k$ we may calculate this ratio. Referring all our thresholds to the new rheobase the results may be re-plotted and a slightly smaller value of λ obtained. The new rheobase

TABLE II—" ACCOMMODATION " (λ) IN THE SCIATIC NERVE OF FROGS

| Frog injected with | Preparation soaked in | λ (m sec) |  | Number of determinations |
|---|---|---|---|---|
|  |  | Average | Limits |  |
| — | Normal Ringer's solution | 18 | 12–22 | 15 |
| — | Unsoaked .............. | 35 | 23–111 | 7 |
| — | Ringer's solution with half normal Ca and K | 35 | 14–91 | 7 |
| $CaCl_2$ | Unsoaked .............. | 10 | 6–18 | 24 |
| — | Ringer's solution with 2–10 times normal Ca | 14 | 10–19 | 10 |
| — | Ringer's solution with 2–4 times normal Ca and K | 13 | 10–25 | 12 |
| Na citrate | Unsoaked .............. | ∞ |  | 5 |

is not the true rheobase, nor is the new λ the true λ, but they are nearer than the original values. Successive approximations may be performed, in this manner, and each will arrive nearer the true values for the rheobase and λ.

Doing two such approximations on the results cited above (taking $k = 0\cdot40$ m sec, which is about the correct value for frog's nerve at 20° C) we find:—

$$\text{Measured rheobase } (I_1) = 1\cdot55$$

$$\lambda = 17\cdot4 \text{ m sec}$$

$$\frac{I_1}{I_0} = \left(\frac{17\cdot4}{0\cdot40}\right)^{\frac{1}{17\cdot4/0\cdot40-1}} = 1\cdot09.$$

$$\text{Hence " true rheobase " } (I_0) - (\text{1st approx.}) = 1\cdot42$$

$$\lambda - (\text{1st approx.}) = 15\cdot5 \text{ m sec}$$

Therefore

$$\frac{I_1}{I_0} = \left(\frac{15\cdot5}{0\cdot40}\right)^{\frac{1}{15\cdot5/0\cdot40-1}} = 1\cdot10,$$

$$\text{and " true rheobase " } (I_0) - (\text{2nd approx.}) = 1\cdot41$$

$$\lambda - (\text{2nd approx.}) = 15\cdot4 \text{ m sec.}$$

For the ordinary range of values of λ and $k$, which yield a value for $\lambda/k$ lying between 40 and 80, the true rheobase is less than 10% lower than the observed (Hill, 1936). For smaller values of $\lambda/k$ the error becomes more serious, reaching 30% at $\lambda/k = 10$. The " accommodation " slopes and values of λ given in this paper have not been corrected for this error; in most cases, as demonstrated, it is not very important. Where its effect is greatest, *i.e.*, in cases of rapid "accommodation", correction results in greater rapidity so the real interest of these cases, which is qualitative, is not vitiated.

Schriever (1932) has defined a time-parameter which he calls the "*Einschleichzeit.*" This is really a measure of the time-constant of "accommodation." It is the time-constant of exponential current rise which will just excite a nerve at a current strength equal to twice the rheobase. Hill (1936) has shown that, if the theoretical equations are obeyed by the experimental data, $\lambda = 2 \cdot 89 \times$ "*Einschleichzeit.*" The theoretical development calls for a straight line only at the longer time-constants of current rise; this straight region is preceded by a curve at the shorter times. This theoretical relationship, with the initial curve followed by a linear relationship, is found in practice only when the preparations have a high λ. In such cases the stated relation between λ and "*Einschleichzeit*" is fairly closely obeyed as shown in Table III (compiled from fig. 4). For nerves, however, with normal or small values of λ the "accommodation" relation is almost invariably a straight line throughout. As a rule, the smaller the value of λ the more nearly perfect the straight line and the more reproducible the data from which it is drawn. This deviation from the theory is not understood. The predicted relation between λ and "*Einschleichzeit*" cannot hold (*see* Table III) where the initial straight-line relationship is found.

TABLE III—RELATION OF λ TO "EINSCHLEICHZEIT"

Compiled from fig. 4

| Relation of $E/E_0$ to RC | λ (m sec) (Reciprocal of slope of straight part of relation) | "*Einschleichzeit*" (RC in m sec at $E/E_0 = 2$) | "*Einschleichzeit*" × 2·89 |
|---|---|---|---|
| (1) Straight line throughout | 7·45 | 7·45 | 21·5 |
| (2) ,, | 16·1 | 16·5 | 47·6 |
| (3) Initial curve at small values of RC, followed by a straight line | 40·0 | 19·0 | 54·9 |
| (4) ,, | 222·0 | 77·5 | 224·0 |
| (5) ,, | ∞ | ∞ | ∞ |

Table II shows that preparations from Winter (November–April) frogs, soaked in normal frog Ringer's solution for more than 1 hour before the determination of $\lambda$, showed an average value of $\lambda = 18$ m sec. Similar preparations with no initial soaking gave an average value of $\lambda = 35$ m sec. Reduction of either or both the $CaCl_2$ and KCl, so that the sum of ionic concentrations of Ca and K was half the normal amount, resulted in a solution which caused no change in the " accommodation " over long periods of soaking.

Preparations taken from frogs soon after the breeding season had an average value of $\lambda = 7 \cdot 4$ m sec (8 determinations). This was raised to $\lambda = 13$ m sec (8 determinations) by soaking for 3 hours in normal frog Ringer's solution. These frogs were very lethargic.

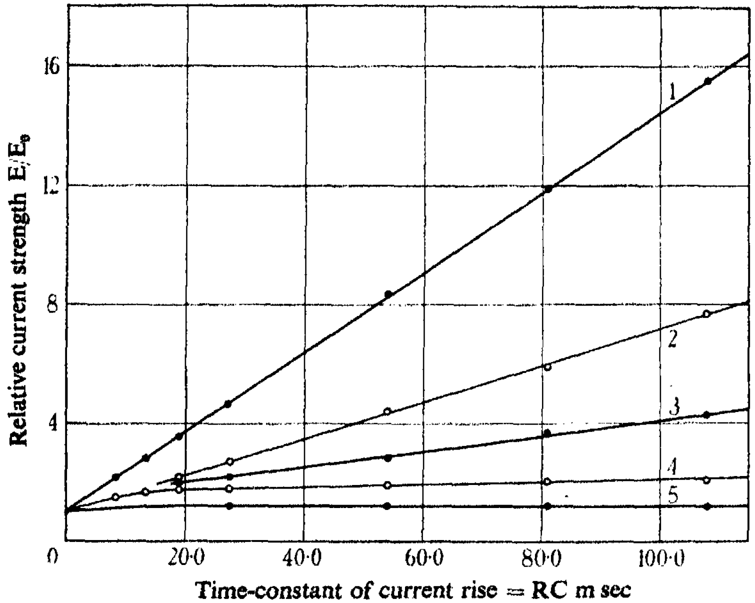


FIG. 4—" Accommodation " curves, sciatic nerves of *Rana esculenta*. Curve 1, preparation from frog injected with 150 mg $CaCl_2$. " Accommodation " slope = 135, $\lambda = 7 \cdot 4$ m sec. 2, preparation from normal (Winter) frog and soaked for 4 hours in normal frog Ringer's solution at 20° C, $\lambda = 16 \cdot 1$ m sec. 3, as 2, but preparation unsoaked. *See also* fig. 5: $\lambda = 39 \cdot 0$ m sec. 4, preparation from frog injected with 100 mg sodium citrate. Temperature = 20° C. *See also* fig. 5. $\lambda = 260$ m sec. 5, as 4, but frog injected with sodium oxalate instead of citrate, $\lambda = \infty$.

It appears that normal frog Ringer's solution contains too much Ca for Winter frogs and too little for Summer frogs. To correct the deficiency for Winter frogs it is probably best to reduce Ca and not K. The concentration of K is less specific in its effect on " accommodation " than that of Ca, as will be shown later. However, both seem effective in reducing λ, so both may be added to Ringer's solution for use with Summer frogs.

A concentrated solution of $CaCl_2$ was injected into the dorsal lymph sac of frogs. Doses of from 20 to 200 mg of the salt in 5 to 10 cc of $H_2O$ were given. After at least 8 hours the frogs were dissected. The average value of λ was found to be 10 m sec (Table II). The group which received over 100 mg of $CaCl_2$ showed $\lambda = 8$ m sec (8 determinations). Soaking preparations from normal Winter frogs for 2 to 6 hours in Ringer's solution containing 2 to 10 times the normal Ca (added as isotonic $CaCl_2$ solution) decreased the value of λ to 14 m sec (8 determinations). Similar decreases in λ were obtained by the addition of $CaCl_2$ and KCl (12 determinations), or KCl alone (4 determinations), in total molecular quantities equivalent to the additions of $CaCl_2$. Injected KCl had an effect similar to injected $CaCl_2$, but was more difficult to work with owing to the lowering of the excitability of the muscle.

Frogs taken soon after the breeding season showed marked lethargy and rapid "accommodation" as previously described. No further lowering of λ could be obtained by injecting $CaCl_2$ or by soaking these preparations in Ringer's solution with added $CaCl_2$.

Preparations showing rapid "accommodation," that is small values of λ, always proved very stable and yielded remarkably reproducible results. Return determinations made on such preparations regularly agreed well with the first series of thresholds obtained. Lucas (1908) observed that soaking frog's nerve-muscle preparations in Ringer's solution containing extra calcium resulted in great stability of the preparation.

Decreasing the ionized Ca in the frogs was attempted by several means. Injections of large doses (up to 200 mg) of sodium oxalate, alkaline phosphate, sodium bicarbonate, or sodium citrate all caused spontaneous twitching. Injecting smaller doses (insufficient to cause the spontaneous twitching) of these substances, preparations with values of λ above 100 m sec were obtained in all cases (10 determinations). Preparations from frogs showing spontaneous twitching due to large doses (200 mg in 5 to 10 cc of $H_2O$) of sodium citrate showed quiescent periods and during these no evidence of "accommodation" could be detected (5 determinations).

Figs. 3, 4, and 5 show "accommodation" curves obtained from normal preparations and those exhibiting the effects of increased and decreased calcium.

It is of interest to note that frogs injected with $CaCl_2$ became lethargic. Although able to jump quite well, they tended to sit sprawled out with their muscles relaxed. Their limbs could, by slow, careful movement, be placed in grotesque positions which the frog would not attempt to correct.

A strong aqueous solution of $MgCl_2$ was injected into the dorsal lymph sac of frogs. Doses of 80 to 180 mg of the salt in 2 to 5 cc of $H_2O$ were given. The frogs took from 12 to 20 hours to become lethargic, when they showed the " accommodative " type of lethargy described in connexion with $CaCl_2$ administration. Preparations from these showed an average value of $\lambda = 12$ m sec (5 determinations). This batch of frogs showed a normal value of $\lambda = 44$ m sec (8 determinations). The experiments were carried out in the early autumn with fresh frogs in good condition. Soaking the preparations for 5 to 10 hours in Ringer's solution containing 100 to 200 mg $MgCl_2$ per 100 cc of solution (added as an isotonic solution) decreased the value of $\lambda$ to 15 m sec (8 determinations). Using much smaller concentrations of $MgCl_2$, or soaking for much shorter times, had little effect on " accommodation." Compared with $CaCl_2$, $MgCl_2$ is somewhat less effective when injected (larger doses required for a comparable change in $\lambda$) and much less effective and slower in action when added to the bathing Ringer's solution.

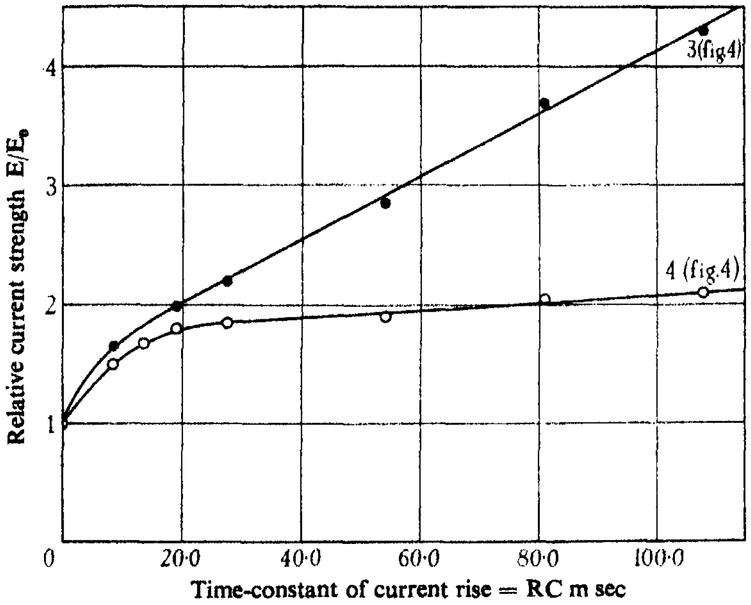


FIG. 5—" Accommodation " curves. Curves 3 and 4 from fig. 4 drawn to larger scale along the ordinate demonstrating the initial curvature as predicted by Hill's theory.

In some situations (Meltzer and Joseph, 1909) calcium opposes the anaesthetic action of magnesium. This was not found to be so with frogs (5 determinations). Furthermore the ions were found to be additive in their effect on "accommodation", whether injected into the frog or added to the Ringer's solution in which the preparations were soaked (6 determinations).

Large doses up to 250 mg of $SrCl_2$ in 5 cc of $H_2O$ were injected into the dorsal lymph sac of frogs. The "accommodation" of the two sciatic nerve preparations from each frog was measured immediately after dissection. One preparation was then soaked in normal Ringer's solution and the other in Ringer's solution with an addition of isotonic $SrCl_2$ solution. This method is useful for detecting slight changes in $\lambda$ due to any particular chemical agent. In this case a decrease in $\lambda$ of 15% due to Sr was observed (4 determinations). Soaking in Ringer's solution with added Sr but no previous injection of Sr had no detectable effect on "accommodation". Injection of up to 250 mg of $BaCl_2$ in 5 cc of $H_2O$ had the same effect as injection of $SrCl_2$ (3 determinations).

It has been shown that small quantities of an acetylcholine-like substance are liberated as an impulse passes along a nerve (personal communication from Professor J. H. Gaddum). The rate of destruction of this substance and the time-constant of "accommodation" might have some connexion. If so a dose of eserine, by prolonging the activity of the acetylcholine-like substance, might alter $\lambda$. Doses of up to 25 mg of eserine sulphate in aqueous solution were injected into the dorsal lymph sacs of frogs. In no case could any change in the $\lambda$ of nerve preparations from these frogs be detected (4 determinations). Soaking normal preparations for up to 6 hours in normal Ringer's solution (buffered to $p_H$ 6·5) with eserine sulphate added to give a concentration of up to 1 : 250,000 also resulted in no change in $\lambda$ (4 determinations).

Injection of large doses (5–40 units) of parathormone (Lilly) caused only a slight decrease in $\lambda$ (5 determinations). Some frogs, kept under observation for 2 weeks after an injection of 25 units of the hormone, appeared quite normal.

Changes in the tonicity (accomplished by changes in the NaCl content), between 0·6% and 0·8% NaCl equivalent, of the Ringer's solution bathing a nerve did not alter the value of $\lambda$ (6 determinations).

Changing the $p_H$ of the Ringer's solution bathing a nerve between 6·4 and 7·8, by means of phosphate buffers, brought about no regular change in "accommodation" (6 determinations). Increasing the $CO_2$ content of the environment is a more effective means of altering the internal $p_H$ of the nerve than soaking in Ringer's solution of the desired

$p_H$. An excitation chamber was constructed so that the nerve of a nerve-muscle preparation could be exposed to $CO_2$ with the muscle in air. Gas mixtures of 5% $CO_2$ in air, of 80% $CO_2$ and 20% $O_2$, and 100% $CO_2$ were used. There was no constant change in λ (8 determinations). In a few cases a 5% decrease, in others a 5% increase was obtained. Two experiments only showed a marked decrease in λ, one of 20% and the other of 50%. This suggests some factors, not controlled in our experiments, involved in the changes in $p_H$, or the effect this has on ionized calcium. The exposure to $CO_2$ always raised the rheobase to 2–4 times its normal value.

Lowering the temperature of normal nerves by 15° C (from approximately 20° C to 5° C) raised λ by 100% (8 determinations). The effect was quantitatively slightly less (80–90%) on preparations which had a low value of λ from treatment with calcium (5 determinations). The quantitative validity of these experiments is questionable, as changes in temperature were quite slow. Owing to alteration of the preparation with time a useful reverse, in respect of temperature, could not be obtained, Schriever's (1932) experiments on changes of " *Einschleichzeit* " with temperature are subject to similar criticism.

Splitting the connective tissue sheath of a nerve caused no change in the value of λ (6 determinations). The effect on λ of soaking in Ringer's solution with added calcium or potassium was not changed by splitting the sheath (4 determinations).

In all these experiments care was taken not to injure the nerve fibres. It has been generally observed throughout all the work on " accommodation " that injury of any sort results in a low value of λ. If a preparation, well soaked in Ringer's solution, is set up on electrodes and values of λ obtained at intervals it will be found that for some time λ remains constant, after a small initial change, and then starts to decrease. Low values of λ are obtained with preparations which are, for any reason, in poor condition. Fabre (1931) noticed that his " *constante linéaire* " changed with time, and to a different degree from the chronaxie.

All the experiments on " accommodation " described were performed with sharp edges of pieces of wood making contact with the nerve. Substituting broad contacts (1 cm wide) had no effect on the value of λ obtained (5 determinations).

The distance between the two points of contact with the nerve was always over 20 mm in the foregoing experiments. In testing the effect of separation of the points of contact on the measurement of λ, small separations were compared with a fixed large separation of 30 mm. At small separations it was difficult to obtain consistent results, as the high currents required seemed to alter the nerve. No regular difference in λ could be found when measured with 6 mm or 30 mm separation. Below 6 mm the value of λ was lowered; the value of λ with 2 mm contact separation was about 75% of its value with 30 mm separation.

### " *Accommodation* " *in Motor Nerves of Fishes*

Table IV is a summary of results. The value of λ in unsoaked segmental motor nerves (nerves innervating the " wing " musculature) of rays was 20 m sec (12 determinations). These preparations were bathed only in the fishes' body fluids. As the value of λ was raised by soaking the nerves in elasmobranch Ringer's solution the effect of adding calcium to the solution was tried. Increasing the calcium content to 4 or 5 times the normal provided a solution which did not alter the value of λ in most preparations. Further increases in calcium, either by soaking the preparation in calcium-rich Ringer's solution or by the intra-peritoneal injection of saturated $CaCl_2$ solution, failed to produce a value of λ outside the normal limits. Table IV shows that these normal limits are from 12 to 140 m sec. This is much wider than in any other form investigated. Our experiments indicate no reason for this wide individual variation. They were performed in September on freshly-caught specimens in good condition. No sex variation was apparent. Both the " spotted ray " (*Raia maculata*) and the " stickleback ray " (*Raia clavata*) were used with similar results.

TABLE IV—" ACCOMMODATION " (λ) IN THE SEGMENTAL MOTOR NERVES OF FISHES

| | Preparation bathed in | λ (m sec) | | Number of determinations |
|---|---|---|---|---|
| | | Average | Limits | |
| Ray (" skate ") *Raia maculata* and *Raia clavata* | Fishes' body fluids | 20 | 12–140 | 12 |
| ,, ,, | Elasmobranch Ringer's solution | 71 | 47–120 | 4 |
| Flatfish (" plaice ") *Pleuronectes platessa* | Teleost Ringer's solution | 11 | 7–25 | 7 |

From Table IV it is apparent that the segmental motor nerves of " plaice " (*Pleuronectes platessa*) show a more rapid " accommodation " than those of the rays, λ being 11 m sec (7 determinations). A deteriorating preparation, as with all other species thus investigated, shows an increasing rapidity of " accommodation." In teleosts the deterioration was very rapid, so it is probable that the values obtained for λ are too low. Successive determinations always resulted in successively lower values of λ, whether with or without bathing in teleost Ringer's solution. These preparations did not remain active long enough to permit satisfactory determinations on the effect of calcium on the value of λ.

### " *Accommodation* " *in the Limb Nerves of Crabs and Lobsters*

The limb nerves in crabs and lobsters have no enveloping connective tissue sheath. They are composed of bundles of strictly non-medullated fibres (Young, 1935). These bundles are held together by connective tissue and each axon is covered with a fairly complete connective tissue sheath. Table V shows that the values of λ in these nerves are much larger than in any other form investigated. In *Maia* the average value of λ is 1670 m sec (24 determinations), and in *Carcinus* it is 4160 m sec (5 determinations). Other crabs and lobsters investigated have nerves showing values of λ lying between these limits. Increasing the calcium content (to 10 times normal) of the sea-water in which the preparations were soaked decreased the value of λ in *Maia* to about 55% of normal (4 determinations). Similar decreases were observed with the other forms but these were not accurately evaluated. Carefully dissected unsoaked preparations gave approximately the same values as those soaked in normal sea-water. Unsoaked preparations tended to become

TABLE V—" ACCOMMODATION " (λ) IN THE LIMB NERVES OF CERTAIN CRUSTACEA

| | Preparation soaked in | λ (m sec) | | Number of determinations |
|---|---|---|---|---|
| | | Average | Limits | |
| Spider crab (*Maia squinado*) | Sea-water ...... | 1665 | 800–3220 | 24 |
| | Sea-water plus 10 times normal Ca | Decreased λ to 55% of the normal value | | 4 |
| Lobster (*Homarus vulgaris*) | Sea-water ...... | 3710 | 2500–7690 | 7 |
| Green shore crab (*Carcinus maenas*) | Sea-water .... | 4160 | 3030–5880 | 5 |
| Edible crab (*Cancer pagurus*) | Sea-water ...... | 3225 | 2630–5000 | 4 |

inexcitable and so were difficult to work with. Crab Ringer's solution (Pantin, 1934) constituted to have the inorganic salt constituents of crab blood, contains about the same amount of calcium as sea-water (0·42 parts in 1000 by weight). We should therefore expect no marked change in " accommodation " due to this factor, after soaking in sea-water.

Soaking preparations from *Maia* in sea-water containing three times the normal $MgCl_2$ produced no constant change in λ (4 determinations).

Many crab nerve-muscle preparations will not respond to a single nerve impulse (Pantin, 1934). This was found to be true for *Carcinus* preparations and for *Maia* preparations which were not in good condition. In either case stimuli to the nerve, which are shorter than the absolute refractory period of the nerve (for *Carcinus maenas* = 1 m sec; for *Maia squinado* = 2 to 3 m sec), no matter how strong, fail to elicit a muscle response. The values of λ cited, for *Carcinus* at least, depend therefore chiefly on " accommodation " but partly also on the tendency to repetitive response on the part of the nerve. Since a minimal twitch was taken as an index of excitation it is probable that this latter factor plays only a small and constant part. To obtain a true measure of " accommodation " in such nerves it would be necessary to use, as a detector of the nerve impulse, not the muscle but a valve amplifier and oscillograph.

TABLE VI—" ACCOMMODATION " (λ) IN HUMAN ULNAR NERVE

| Subject | λ (m sec) Average | λ (m sec) Limits | Number of determinations |
|---|---|---|---|
| Male .................. | 59 | 42–83 | 13 |
| Female ................ | 56 | 50–67 | 3 |
| Male (sodium bicarbonate given by mouth 1 hour before these values obtained) | — | 100 and 140<br>(normal values for these subjects at this time lay between 55 and 60) | 2 |
| Male (ammonium and calcium chloride given by mouth 2 hours before these values obtained) | 40 | 37–45<br>(normal values for these subjects at this time lay between 55 and 60) | 4 |

### " *Accommodation* " *in the Ulnar Nerve of Man*

The values for λ in healthy men (13) and women (3) lay between 42 and 83 m sec with an average of 58 m sec. Further details are given in Table VI. No significant sex difference was observed, although the number of experiments performed on women was too small to permit a definite conclusion on this point.

Fig. 6 shows “ accommodation ” curves drawn from data on normal male subjects. The initial curvature preceding the linear relation is well illustrated in (1) and is detectable in (2). In some cases the linear relation is found throughout.

The results obtained on the same subject varied only slightly from hour to hour or from week to week. The value of λ on one subject remained between 42 and 55 m sec over a period of one month during which repeated readings were made.

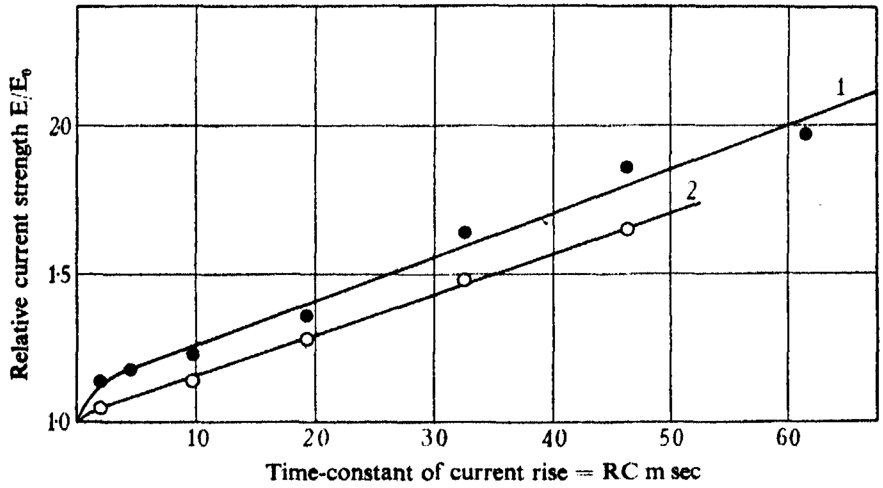


FIG. 6—“ Accommodation ” curves. Human ulnar nerve. 1, normal male subject, λ = 67 m sec; 2, normal male subject, λ = 72 m sec.

Attempts were made to alter the quantity of ionized calcium in the blood, so that the effect on “ accommodation ” could be measured. The value of λ was obtained every half-hour during the experiment. After the first two determinations 60 gm of sodium bicarbonate in water was administered by mouth. The maximum effect was present about 1 hour after the alkali was taken. A subject with a normal value of λ = 70 m sec showed an increase to λ = 140 m sec. This latter represents slower “ accommodation ” than was ever found on a normal subject. Presumably the bicarbonate, by producing an alkalosis, decreases the quantity of ionized calcium in the blood. This effect of sodium bicarbonate on the “ accommodation ” was observed on four occasions, although careful measurements of λ were only carried out on two of these subjects.

Doses of $CaCl_2$ (10 gm) and $NH_4Cl$ (10 gm) in water were given both separately and together. These substances tended to produce nausea, but two subjects on two occasions retained the doses administered. In both a marked decrease of λ was obtained. Normal values of $\lambda = 55$–$60$ m sec were reduced to $\lambda = 37$–$45$ m sec, the effect being most marked 1 to 3 hours after the salts were administered. Here we find the effect on " accommodation " which one would predict for an increase in the ionized calcium produced in this case by an artificially induced acidosis.

It is of interest that two clinical cases showing low diffusible blood calcium were available for the experimental measurement of λ. One of these, a woman, suffered from persistent vomiting which had induced a rather severe alkalosis. Chvostek's and Trousseau's signs were present. The value of λ was repeatedly found to be around 140 m sec. The other patient, a boy, suffered from hypo-parathyroidism and also showed the clinical signs associated with low ionized calcium. The value of λ in this case was about 160 m sec. In both patients " accommodation " was much slower than ever observed in the normal person. The woman recovered when the therapeutic measures for stopping the vomiting proved successful, and her value of λ rapidly came within normal limits. Unfortunately it was impossible to investigate " accommodation " in the boy during or after recovery.

## DISCUSSION

The foregoing experiments were performed to demonstrate the existence of a time-constant of " accommodation " by means of excitation with exponentially increasing currents in the manner outlined by Hill (1935 and 1936). One of the objects was to test Hill's theoretical development of this second time-constant of electrical excitation. The theory predicts a linear relation between the relative threshold and the time-constant of current rise for values of the latter which are large compared with the $k$ of the tissue in question. The experimental results fully confirm this part of the theory. Data on the nerves of frogs, fishes, crustaceans, and man all yield straight lines over the predicted range.

Cardot and Laugier (1913) performed a few experiments on frogs' (*Rana fusca*) sciatic nerves. They plotted absolute thresholds against time-constants of current rise and obtained good straight lines. The values of λ calculated from their results lie between 8 and 16 m sec, values quite in accord with those cited in the present paper. In later work Laugier (1921) obtained data which do not show the linear relation.

Possibly this is explained by the fact that good non-polarizable electrodes were not used in the later experiments.

Schriever (1930) found a striking linearity between the threshold to an exponentially increasing current and the time-constant of current rise. Delville (1934) performed similar experiments using chlorided silver wires as electrodes. Examination of his data shows a good linear relation when he used no shunt across his preparation.

Hill's theory (1936, fig. 10) predicts a deviation from the linear relation at the shorter time-constants of current rise. Such a deviation is found to occur in most normal frog, crab, and lobster nerve preparations and in many normal human ulnar nerves. The more rapidly " accommodating " nerves, however, of all species investigated show a linear relation throughout. The reason for this deviation from the theory is not understood. Owing to the deviation, the more rapid the " accommodation " the poorer the agreement between Schriever's (1932) " *Einschleichzeit* " and λ as measured from the final slope of the linear relation. The fact that the measurement of " *Einschleichzeit* " depends on the form of the " accommodation " curve in the region where unaccountable variability is found, seriously interferes with Schriever's method as a means of measuring λ.

The predictions of Hill's theory having been, in the main, fulfilled by the experiments, it is reasonable to use exponentially rising currents for obtaining a measure of λ in the manner described. This was done on the nerves of a variety of animals. Not only were the experimental data reproducible on each preparation at the time, but the values of λ obtained on any one type of preparation under one set of conditions were fairly constant.

The variation between preparations of the same type under the same environmental conditions are indicated by the " limits " listed in Table II. The spread of values in the normal frogs suggests that the level of ionized calcium varies from individual to individual. Finding this variation in the normal we should expect to find it also in the treated frogs. In some cases calcium was presumably being injected into frogs with a high level of ionized calcium, and in other cases into frogs with a low ionized calcium. The marked seasonal variation of λ suggests a seasonal variation in ionized calcium. In man, where the level of ionized calcium is constant, the week-to-week values of λ on any one normal individual are also constant.

The values for λ obtained on frogs' nerves under normal conditions were larger than those calculated from Lucas's (1907) or Schriever's (1930 and 1932) data, but agree quite well with those calculated from data cited by Cardot and Laugier (1913). Delville (1934) gives experimental data which yield intermediate values of λ. Lucas soaked all his preparations in Ringer's solution with a high calcium concentration because he found such treatment made the preparations more stable. This would account for the low values of λ calculated from the data he obtained.

Possibly Schriever treated his preparations in a manner similar to that followed by Lucas. He does not give details of the solutions used. In making his determinations Schriever used porcelain-boot electrodes. The foregoing experiments show that the width of contact of these electrodes would not account for the low values of λ obtained. Leakage of $ZnSO_4$ from such electrodes is always a possibility. Exposing a nerve, however, to Ringer's solution contaminated with this salt was found to raise and not to lower λ.

Schriever (1930) and Liberson (1934) have both obtained " accommodation " curves on human nerve in much the same manner as outlined in the present paper. They obtained good straight line relations, at all but very long time-constants of current rise. The values of λ calculated from both these sets of data agree very closely with the present values.

Nerves were exposed to various agents to discover the conditions affecting λ. It was found that the quantity of calcium present had a large effect on λ; the more calcium present the faster the " accommodation", the smaller λ. Indeed λ could be varied, in frog's nerve at room temperature, from 6 m sec to infinity, in a preparation having a normal value in the vicinity of 30 m sec, by altering nothing but the calcium in the environment of the nerve. Blumenfeldt (1925) has shown that the effect of calcium on chronaxie is not of this order, even when the calcium concentrations are double those ever used to test the effect on λ. These facts indicate that λ cannot be considered as a function of $k$; λ and $k$ must be independent time-constants. Schriever (1930 and 1932) varied the resistance of his circuit, the distance between electrodes, and the temperature, and always found the "*Einschleichzeit*" and chronaxie varying in the same direction. He concluded that he had no evidence to show that the one time-constant was not a function of the other. The effect of calcium provides this evidence.

The effect of calcium on λ is apparently specific, although some of its properties are shown by potassium and magnesium and to a lesser degree by strontium and barium. The injection of KCl is about as effective as that of $CaCl_2$ in lowering λ. The presence of normal K in the Ringer's solution will not, however, prevent the absence of calcium from raising λ to a very high value (infinity in some cases). The presence of magnesium lowers $\lambda$, but does this more slowly than calcium. It is interesting to observe that magnesium has no such effect on $\lambda$ in crustacean nerve which is normally exposed to a high concentration of this ion. Here, where calcium and magnesium are both present normally, it is only the former which can be made to demonstrate any controlling influence on " accommodation."

Tipton (1934) has shown that about 60% of the calcium in frog's sciatic nerve cannot be removed by bathing in calcium-free solutions. He believes that this represents intra-cellular calcium. This would indicate that the changes in calcium, which are effective in altering $\lambda$, affect only the extra-cellular calcium content of the nerve. The action of calcium, as demonstrated in the experiments outlined in the present paper, must therefore be on the surface of the nerve fibre. It is an interesting fact that the effect of calcium on $\lambda$ is much less in the crab's non-medullated nerve than in the frog's medullated nerve.

There are many ways in which the " accommodation " of an excitable tissue may be measured. Lucas (1910), for instance, was really dealing with " accommodation " when he measured his " summation interval." Fabre (1927, *a*, *b*) also was actually determining the speed of " accommodation " when he measured the thresholds for various rates of rise of linearly increasing currents. He pointed out the existence of a " *constante linéaire* ", the first mention of a time-constant of " accommodation ", and later (1931) concluded, contrary to his original opinion, that it is independent of chronaxie. In all the experiments outlined in the present paper " accommodation " has been measured with exponentially increasing currents because this is technically the simplest method. Hill (1936) has developed the theoretical treatment of a number of methods by which $\lambda$ may be measured. In a later paper it is hoped to give an experimental comparison of these.

The experimental work outlined in this paper is based on theory developed by Professor A. V. Hill. The author is indebted to Professor Hill for kindly encouragement and wise counsel during the work, and for permission to include the results of some of his experiments. The experiments on fishes and crustacea were performed at the Marine Biological Laboratory, Plymouth, and it is a pleasure to thank Dr. E. J. Allen for the excellent facilities provided. Mr. J. L. Parkinson's technical assistance has been invaluable throughout. Special thanks are due to him for acting as subject of some of the more unpleasant experimental procedures.

## SUMMARY

Experiments are outlined by which Hill's (1935 and 1936) theory concerning the time-constant of "accommodation" in the electrical excitation of nerve is verified. The predicted linear relation between relative threshold and time-constant of exponential rise of current was found for certain motor nerves of frogs, fishes, crabs, lobsters, and man.

The slope of this line has been shown by Hill to be the reciprocal of $\lambda$, the time-constant of "accommodation". Measures of $\lambda$ were thus obtained on a variety of nerves under various conditions. The sciatic nerves of normal Winter frogs (*Rana esculenta* and *Rana temporaria*) showed an average value of $\lambda = 35$ m sec at room temperature. Fish had somewhat larger values of $\lambda$, averaging between 11 and 20 m sec. Crabs and lobsters had values of $\lambda$ between 800 and 7690 m sec. The average value of $\lambda$ for the human ulnar nerve was 58 m sec.

Increasing the concentration of calcium or potassium around a frog's nerve, either by injecting the chloride into the animal, or by adding it to the Ringer's solution used to soak the preparation, was found to lower $\lambda$. In the case of normal Winter frogs injected with more than 100 mg of $CaCl_2$, $\lambda$ was as small as 6 m sec. Magnesium acted similarly but more slowly, while strontium and barium also had the same effect but to a lesser degree. Increasing the ionized calcium concentration in the environment of a crab's nerve, or of the human ulnar nerve, was found to lower $\lambda$.

Decreasing the calcium ion concentration in the environment of frog's sciatic nerve was found to raise $\lambda$ till, in the absence of calcium, it approached infinity. No other treatment (except a lowering of temperature) was found by which $\lambda$ could be increased. Decreasing the calcium-ion concentration in man, by the ingestion of alkali, increased the value of $\lambda$.

The effect of calcium on $\lambda$ is much greater than on $k$. This shows that the time-constants of "accommodation" and of "excitation" are independent.

## REFERENCES

Blumenfeldt, E. (1925). 'Biochem. Z.,' vol. **156**, p. 236.

Cardot, H., and Laugier, H. (1913). 'J. Physiol. Path. gén.,' vol. **15**, p. 1134.

Delville, P. (1934). 'Arch. int. Physiol.,' vol. **40**, p. 83.

Fabre, P. (1927, *a*). 'C.R. Acad. Sci., Paris,' vol. **184**, p. 1486.

—— (1927, *b*). *Ibid.*, vol. **185**, pp. 300, 1216.

—— (1931). 'C.R. Soc. Biol., Paris,' vol. **108**, pp. 1248, 1250.

Hill, A. V. (1935). " Advances in Modern Biology," Moscow, vol. **4**, p. 131.
—— (1936). ' Proc. Roy. Soc.,' B, vol. **119**, p. 305.
Lapicque, L. (1908). ' C.R. Soc. Biol., Paris,' vol. **64**, p. 336.
—— (1926). " L'excitabilité en fonction du temps," Presses Universitaires, Paris.
Laugier, H. (1921). ' Thesis,' Paris, Imprimerie Paul Marmy.
Liberson, W. (1934). ' C.R. Soc. Biol., Paris,' vol. **116**, p. 1319.
Lucas, K. (1907). ' J. Physiol.,' vol. **36**, p. 253.
—— (1908). *Ibid.*, vol. **37**, p. 459.
—— (1910). *Ibid.*, vol. **39**, p. 461.
Meltzer, S. J., and Joseph, D. R. (1909). ' J. Pharmacol.,' Baltimore, vol. **1**, p. 1.
Nernst, W. (1908). ' Pflügers Arch.,' vol. **122**, p. 275.
Pantin, C. F. A. (1934). ' J. exp. Biol.,' vol. **11**, p. 11.
Schriever, H. (1930). ' Z. Biol.,' vol. **91**, p. 173.
—— (1932). *Ibid.*, vol. **93**, p. 123.
Tipton, S. R. (1934). ' Amer. J. Physiol.,' vol. **109**, p. 457.
Young, J. Z. (1933). ' Pubbl. Staz. Zool. Napoli.,' vol. **12**, p. 425.
—— (1935). ' J. Physiol.,' vol. **85**, p. 2P.

(*Abstract*)

612 . 824 . 1 : 544 . 62

# Quantitative Spectrographic Analysis of Biological Material. I—A Method for the Determination of Lead in Cerebrospinal Fluid

By J. S. FOSTER, D.Sc., F.R.S., Professor of Physics, G. O. LANGSTROTH, Ph.D., and D. R. MCRAE, Ph.D., Research Associates, McGill University

(*Received August* 14, 1935)

A quantitative spectroscopic method of the determination of small concentrations of lead in certain biological material is described. It is valid for concentrations between $1 \times 10^{-8}$ and $2 \times 10^{-5}$ gm/cc, and the precision of a determination is better than 15%.

The ability to make determinations with very small samples, and to use the material without previous treatment, are important features of the method.

The general considerations on which this type of analysis is based are discussed.

(*The full paper is published in* '*Proc. Roy. Soc.*,' *A, vol.* 153, *p.* 141 (1935).)

612.744.2:612.015.3

# The Effects of Prolonged Muscular Exercise on the Metabolism

By F. C. COURTICE and C. G. DOUGLAS, F.R.S.

(*Received October* 10, 1935)

## INTRODUCTION

During recent years a great deal of attention has been devoted to the changes in metabolism which result from very hard muscular work, in the main with the idea of correlating the alterations in the respiratory exchange and in the composition of the blood with the chemical changes responsible for the energy of muscular contraction in the isolated muscle under both aerobic and anaerobic conditions. This work, valuable though its results have been, has tended to divert attention away from another aspect of muscular work which is none the less of great practical importance. In everyday life the muscular work which we are called upon to perform is done at a far lower rate than that which is done by the athlete who is pushing himself almost to the limit of his power. It by no means follows that the facts observed during severe exercise, and the deductions made from these, can be applied without alteration when we wish to explain the effects of exercise of far less severity.

It is true that there have been in the past many studies of the physiology of moderate muscular work, but when these are examined in detail they prove to be rather incomplete. As a rule those who have undertaken these investigations have devoted themselves to some limited aspect of the problem, such as the efficiency with which the work is performed under a variety of conditions, the influence of alterations of the diet on the capacity of an individual to do muscular work, or the vexed question of the relative importance of carbohydrate and fat as sources of energy during the performance of the work, and whilst much stress has been laid on events during the actual work too little attention has been given to changes which may result from the work and become apparent during a subsequent period of rest. It therefore seemed to us desirable to study in more detail the effects of prolonged though moderate muscular work, in the hope in particular of throwing further light on the significance of changes of the respiratory quotient, the interpretation of which is admittedly always a very difficult and troublesome problem, as may be gathered from the observations of Cathcart and Markowitz (1927) and the recent review of Rapport (1930), although it is one that must be faced if we are to gain an insight into the metabolic changes involved.

On consideration we felt that the most profitable course to pursue would be to choose a form of muscular exertion that we could maintain without difficulty for a considerable length of time in the hopes that the total energy expenditure involved would entail a material reduction of what one may call, in general terms, the readily available stores of carbohydrate. If the investigation were not limited to the actual period of the work, but were extended to cover a period of an hour or two of subsequent rest, we anticipated that a review of the whole course of events would assist us in our interpretation of the results. If the observations are restricted to the period of work a difficulty is at once encountered, since, to permit of the observations, the work may have to be done on some form of stationary ergometer, a thing to which the subject may be unaccustomed. Our desire was, however, to study the behaviour of a normal average individual when doing a type of work natural to him. For this reason we decided to abandon the ergometer and substitute walking as the exercise.

Strenuous muscular exercise leads, as is well known, to the appearance of excessive amounts of lactic acid in the blood owing to the fact that the muscular work is in part anaerobic, and as the lactic acid accumulates in the blood during the exercise and is eliminated again in a subsequent period of rest there are resultant variations in the respiratory quotient which are liable to confuse the issue when this quotient is to be used as an index of metabolic changes of a different type. Bock and others (1928) have, however, shown that in a really highly trained athlete a surprising amount of muscular work per minute can be accomplished without the appearance of a significant excess of lactic acid in the blood, whilst Owles (1930) has shown that even in the untrained person a certain critical rate of muscular work must be exceeded before excess lactic acid appears in the blood, a result later confirmed by Cook and Hurst (1933).

Owles's observations were particularly relevant to the present investigation since many of them were made on Douglas, and he found that when walking the critical level corresponded to an oxygen consumption of about 1·8 litres per minute, implying a pace of about 4·5 miles per hour. We felt therefore that so long as we did not exceed this pace we should reduce to a minimum, if not abolish entirely, the effects attributable to lactic acid accumulation and disappearance and in this way simplify the problem.

It was clear that if we were to get any substantial changes as a result of the walk we should have to continue the exercise for a considerable time. At the same time it was expedient to walk as fast as we dared without running the risk of encountering the lactic acid difficulty so that the whole experiment could be finished in a reasonable time, for it must be remembered that an essential part of our investigation was to continue our observations during a period of several hours' rest after the walk was over. We decided therefore to adopt as our standard exercise a walk on the flat of 10 miles (16 kilometres) at a pace of 4·5 miles (7·25 kilometres) per hour, the walk lasting therefore for just under 2¼ hours. In some of the experiments we modified the speed and duration of the walk, and in others we repeated the walk after an interval of rest for a further 6 miles at the standard rate. Many of the experiments were made in the post-absorptive state so as to avoid the influence of food absorbed from the alimentary canal, but as time went on we took food, and in particular carbohydrate, at various times preceding the experiment as this seemed likely to give us additional information of value. In the post-absorptive experiments although no food was taken fluid was allowed. Douglas took two large cups of tea, and Courtice a glass of water, about 20 minutes before the experiment began, and both subjects often drank water at the end of the walk. We did this deliberately so as to ensure a reasonably free secretion of urine throughout the experiment since urine analyses were necessary. This ingestion of fluid explains the fluctuations of urine secretion, and especially the early diuresis, which will be noticed when the results are described.

We ourselves served as the subjects of experiment, differing considerably in age and build; Courtice, aged 24 years with a height of 5 feet 7½ inches, weighing 144 pounds, and Douglas, aged 52 years with a height of 5 feet 10½ inches, weighing 175 pounds, these weights including the clothes in which we walked. We must emphasize the fact that we were in no sense in athletic training; this is certainly true of Douglas, though Courtice was undoubtedly in the better physical condition as he did at intervals play games. As a rule we acted alternately as subjects, and as several days elapsed before the same subject repeated the walk again we do not think that our experiments in themselves led to any material muscular training.

## METHODS

For the purpose of the walks we used two measured tracks on gravel paths. The larger was 1·39 miles (2,237 metres) in circumference, and roughly square in shape. The east and west sides were level, the south side had a very slight gradient down-hill, the north side a correspondingly slight gradient up-hill. The smaller track, round the laboratory buildings, was 269 yards (246 metres) in circumference and level. In order to maintain a pace of 4·5 miles per hour the larger track has to be covered in 19 min 30 sec, the smaller in 2 min 2 sec. It was a little difficult to keep the pace quite steady on the large track, but deviations from the required figure were on the average trivial. On the small track, however, there was less difficulty as it was easy to check each circuit against the watch.

The course of a full experiment was as follows. On arriving at the laboratory the subject reclined at rest in a deck chair. Half an hour later his resting respiratory exchange was determined by the bag method over a period of about 10 minutes, and as a rule a second duplicate determination was made in the next 10 minutes. He then started on his walk round the large track. On returning to the laboratory he paused just long enough to allow the bag apparatus to be slipped onto his shoulders and at once continued his walk round the small track. During the third and fourth circuits of this track he collected a sample (80–100 litres) of his expired air, taking the time with a stop watch. On completing the fourth circuit he handed over the bag apparatus to his colleague and set off again round the large track. This procedure was repeated until he had completed five circuits of the large track and 4 × 5 circuits of the small track. He then went back into the laboratory and sat down again in the deck chair until the end of the experiment. During this resting period the total respiratory exchange was determined at half-hourly intervals over a period of 8–10 minutes. In some of the experiments a smaller number of samples of expired air was collected during the walk, but the total distance walked was kept the same. Thus if three samples only were collected the small track was only covered 12 times in all and an extra circuit of the large track was added to make up the 10 miles.

The bladder was emptied just before beginning the walk and just after ending it, and further samples of urine were taken at intervals during the ensuing period of rest. Samples of alveolar air and blood were also collected when required before and at various times after the walk.

### *Analytical Methods*

(1) The total respiratory exchange was determined in the usual way by the bag method. In order to minimize any error caused by absorption of $CO_2$ by the rubber the bags were always kept partly filled with expired air in the intervals of use, and we satisfied ourselves that under these conditions a sample of expired air did not change materially in

composition when left in the bag for over half an hour. We always took the precaution of measuring the contents of the bag, and taking a sample for analysis, as soon as possible after the bag had been filled by the subject during an experiment.

(2) The alveolar air was taken during rest by the Haldane-Priestley method. In order to reduce the risk of error without multiplying the number of analyses the mercury with which the gas sampling tube had been filled was allowed to run out in three equal fractions; three samples of alveolar air could thus be collected in a single tube. The figures given in the tables represent the mean of alveolar samples obtained at the end of three normal inspirations and of three normal expirations.

(3) Body temperature was taken in the rectum or mouth with ordinary clinical thermometers.

(4) Blood required for the quantitative determination of $CO_2$ combining power, lactic acid, and ketone bodies was taken with a syringe from a vein in the arm after free circulation has been encouraged by immersing the hand and forearm in warm water for 2 or 3 minutes. Coagulation and glycolysis were prevented by the addition of 0·2% potassium oxalate and 0·05% neutral sodium fluoride (in later experiments increased to 0·1%). The blood was immediately put into a tube sunk in melting ice in a Thermos flask and kept here until it was required for use.

The $CO_2$ combining power of the blood was determined in duplicate at about 41 mm $CO_2$ pressure and 37° C by the method detailed by Douglas and Priestley (1924) using the blood gas apparatus designed by Haldane (1920), the latter being immersed in a water bath maintained at 20° C.

Lactic acid in the blood was determined by the method of Friedemann, Cotonio, and Shaffer (1927), phosphoric acid being substituted for sulphuric acid (Friedemann and Kendal, 1929). In other details we followed Owles's procedure. We wish to thank Dr. R. B. Fisher for the loan of the necessary apparatus. Control analysis made on solutions of lithium lactate in which the concentration of lactate was about the same as that found in the blood of the resting subject gave an average recovery of 97%. Duplicate analyses employing two different sets of apparatus were always made, and the titrations usually agreed within 0·02 cc N/200 iodine, and always within 0·05 cc.

The total ketone bodies in the blood and urine were determined by the gravimetric method of Van Slyke (1917). In this method β-hydroxybutyric acid and aceto-acetic acid are oxidized to acetone and the latter is precipitated in combination with mercuric sulphate (Denigès, 1898). We have expressed our results in terms of β-hydroxybutyric acid: we made no attempt to estimate the ketone bodies individually. Owing to the quantity of blood or urine required it was only possible to do a few of these analyses in duplicate.

Total nitrogen in the urine was estimated in duplicate by the micro-Kjeldahl method. The urine for these analyses was preserved under toluol.

Blood sugar was estimated in samples of capillary blood obtained from the finger by puncture. The method used was that of Hagedorn and Jensen as given by Peters and Van Slyke (1932), and the analyses were done in duplicate.

All analyses were done by Courtice.

## RESULTS OBTAINED ON THE POST-ABSORPTIVE SUBJECT WHO HAD PREVIOUSLY BEEN TAKING HIS NORMAL DIET

The results of typical experiments on each of the subjects are shown in Tables I and II. In these five determinations of the respiratory exchange were made during the work and six during the subsequent rest period at half-hourly intervals. It will be seen that Douglas walked at slightly above the standard pace. Figs. 1 and 2 show two further experiments in which the respiratory exchange was followed continuously during the first half-hour after stopping the work by using four bags in succession.

TABLE I—DOUGLAS, POST-ABSORPTIVE. EFFECT OF 10-MILE WALK AT 4·6 MILES PER HOUR

| | Respiratory exchange cc/min | | R.Q. |
|---|---|---|---|
| | $CO_2$ | $O_2$ | |
| Rest before walking | 179 | 236 | 0·76 |
| End of 2 miles' walk | 1504 | 1851 | 0·81 |
| „ 4 „ | 1375 | 1742 | 0·79 |
| „ 6 „ | 1476 | 1832 | 0·81 |
| „ 8 „ | 1435 | 1834 | 0·78 |
| „ 10 „ | 1490 | 1889 | 0·79 |
| Rest ½ hour after walk | 181 | 255 | 0·71 |
| „ 1 „ | 177 | 244 | 0·72 |
| „ 1½ „ | 175 | 237 | 0·74 |
| „ 2 „ | 177 | 238 | 0·74 |
| „ 2½ „ | 170 | 238 | 0·72 |
| „ 3 „ | 184 | 246 | 0·75 |

Table II—Courtice, Post-absorptive. Effect of 10-mile Walk at 4·5 Miles per Hour

| | Respiratory exchange cc/min | | R.Q. |
|---|---|---|---|
| | $CO_2$ | $O_2$ | |
| Rest before walking | 182 | 218 | 0·84 |
| End of 2 miles' walk | 1399 | 1645 | 0·85 |
| ,, 4 ,, | 1360 | 1623 | 0·84 |
| ,, 6 ,, | 1454 | 1703 | 0·85 |
| ,, 8 ,, | 1404 | 1697 | 0·83 |
| ,, 10 ,, | 1450 | 1749 | 0·83 |
| Rest ½ hour after walk | 183 | 237 | 0·77 |
| ,, 1 ,, | 178 | 237 | 0·75 |
| ,, 1½ ,, | 181 | 236 | 0·77 |
| ,, 2 ,, | 187 | 236 | 0·79 |
| ,, 2½ ,, | 178 | 236 | 0·76 |
| ,, 3 ,, | 184 | 238 | 0·77 |

It should be noted that in fig. 2 the pace maintained by Douglas is 4·75 miles per hour. In our earlier experiments Douglas tended to walk faster than the rate which we later adopted as a standard.

The general course of events can be at once appreciated in these tables and figures. Starting with a normal post-absorptive respiratory exchange and quotient at rest the respiratory exchange rises during the work to a level which is maintained fairly steady so long as the work lasts, while the respiratory quotient tends to rise above the original resting level at the commencement of the work, and, as the work continues, either remains fairly steady at this figure or shows a gradual fall. When the work stops the respiratory exchange falls rapidly and after the first half-hour there is very little further change during the remainder of the experiment. The respiratory quotient, however, falls to a figure definitely below the initial resting value and remains low for the whole 3 hours' rest subsequent to the work.

Individual experiments made on different days naturally showed variation in detail though these variations were not large. As these experiments were made in precisely the same way, and the bag samples were collected at the same intervals of time, a fairer picture of the behaviour of the respiratory exchange can be obtained by averaging a number of experiments on each subject. Owing to the fact that, as will be seen later, we had a number of other observations to make in addition to those on the respiratory exchange we were often obliged to curtail

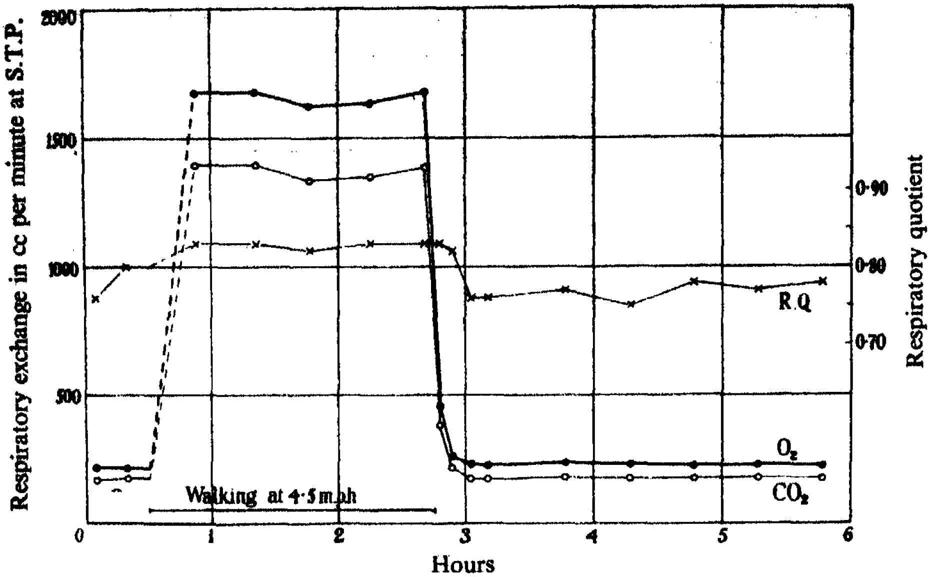


FIG. 1—Courtice, post-absorptive. Effect on respiratory exchange and respiratory quotient of a 10-mile walk at 4·5 miles per hour. × R.Q.; ● $O_2$; ○ $CO_2$

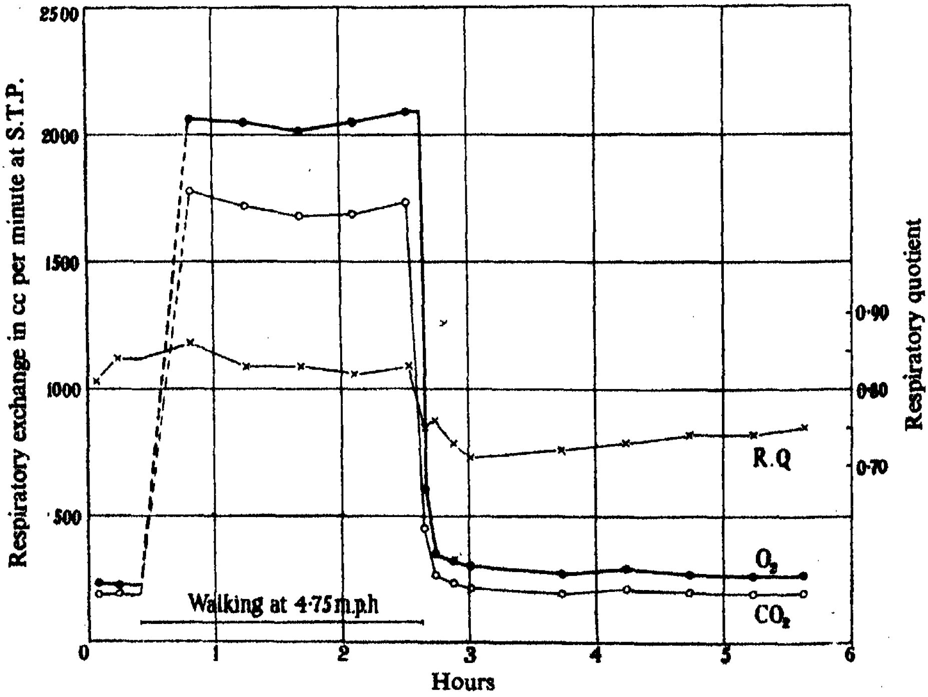


FIG. 2—Douglas, post-absorptive. Effect on respiratory exchange and respiratory quotient of a 10-mile walk at 4·75 miles per hour. × R.Q.; ● $O_2$; ○ $CO_2$.

the number of bag samples taken either during the work period or during the subsequent rest period. We have therefore only a limited number of experiments in which five determinations of the respiratory exchange were made during the walk and six at intervals of half an hour in the period of rest after the walk. On the other hand there is a far larger number of figures available for determining the average pre-exercise respiratory exchange.

Table III gives the average figures for each subject for the respiratory exchange when resting in the post-absorptive state before beginning the exercise. The values (*a*) and (*b*) were obtained during two consecutive periods of approximately 10 minutes' duration after a preliminary period of half an hour's rest.

TABLE III—AVERAGE PRE-EXERCISE RESPIRATORY EXCHANGE WHEN SITTING AT REST POST-ABSORPTIVE

| | Respiratory exchange cc/min | | R.Q. |
|---|---|---|---|
| | $CO_2$ | $O_2$ | |
| *Courtice* | | | |
| Average of 35 experiments from October 24, 1933, to December 10, 1934— | | | |
| (*a*) | 179 | 225 | 0·80 |
| (*b*) | 182 | 221 | 0·82 |
| *Douglas* | | | |
| Average of 17 experiments from October 21, 1933, to November 15, 1934— | | | |
| (*a*) | 182 | 232 | 0·78 |
| (*b*) | 184 | 229 | 0·80 |

It will be seen that Douglas gives slightly higher figures for the respiratory exchange than Courtice, no doubt because of their difference in size, and a slightly lower respiratory quotient. The second of the two determinations gives a slightly higher quotient than the first in each case. Why this should be so we cannot explain, but we satisfied ourselves that further prolongation of the rest does not lead to any sensible alteration in either the respiratory exchange or respiratory quotient. This is shown in Table IV which gives the figures obtained for each subject when the respiratory exchange was determined during rest at half-hourly intervals for a period of 3 hours.

We have seven experiments on Courtice and four on Douglas in which five determinations of the respiratory exchange were made during the

Table IV—Respiratory Exchange with Subjects at Rest, Post-absorptive, without Exercise. Rest started at 9.00 a.m.

| Time | Courtice | | | Douglas | | |
|---|---|---|---|---|---|---|
| | Respiratory exchange cc/min | | R.Q. | Respiratory exchange cc/min | | R.Q. |
| | $CO_2$ | $O_2$ | | $CO_2$ | $O_2$ | |
| 9.30 a.m. .... | 191 | 227 | 0·84 | 183 | 230 | 0·80 |
| 10.00 „ .... | 195 | 231 | 0·84 | 180 | 231 | 0·78 |
| 10.30 „ .... | 194 | 230 | 0·85 | 177 | 224 | 0·79 |
| 11.00 „ .... | 184 | 225 | 0·82 | 169 | 221 | 0·77 |
| 11.30 „ .... | 191 | 225 | 0·85 | 174 | 222 | 0·78 |
| 12 noon ...... | 185 | 224 | 0·83 | 181 | 232 | 0·78 |

walk. In the experiments on Courtice the pace was in each instance 4·5 miles per hour. With Douglas two experiments were made at this pace and two at the pace of 4·75 miles per hour.

Table V gives the average figures for each subject in these experiments.

Table V—Average Respiratory Exchange during Exercise. Subjects Post-absorptive

| Time | Courtice | | | Douglas | | |
|---|---|---|---|---|---|---|
| | Respiratory exchange cc/min | | R.Q. | Respiratory exchange cc/min | | R.Q. |
| | $CO_2$ | $O_2$ | | $CO_2$ | $O_2$ | |
| Rest before walk | 181 | 225 | 0·80 | 189 | 237 | 0·80 |
| End of 2 miles.. | 1359 | 1638 | 0·83 | 1547 | 1841 | 0·84 |
| „ 4 „ .. | 1331 | 1622 | 0·82 | 1507 | 1840 | 0·82 |
| „ 6 „ .. | 1328 | 1631 | 0·81 | 1525 | 1863 | 0·82 |
| „ 8 „ .. | 1310 | 1632 | 0·80 | 1528 | 1881 | 0·81 |
| „ 10 „ .. | 1325 | 1658 | 0·80 | 1534 | 1901 | 0·81 |
| Rest after walk | 181 | 239 | 0·76 | 185 | 249 | 0·74 |

The initial figures for rest agree very closely with the general average figures already given in Table III. The respiratory exchange for Douglas during the walk is higher than for Courtice on account of the slightly faster rate at which he walked and of his greater weight, and the oxygen consumption shows a tendency to rise in the latter half of the walk. The same tendency was shown in other experiments in which fewer determinations were made during the course of the walk. It is difficult to say that Courtice shows a similar phenomenon in the figures given in Table V, but in some individual experiments he, too, had an oxygen consumption which was somewhat greater at the end of the walk than it had been in the earlier stages. This change, when it occurs, may possibly be due to fatigue.

The respiratory quotient shows a perfectly definite variation. After walking 2 miles the quotient, which at rest previously was 0·80, is 0·83, and 0·84 respectively in the two subjects. It subsequently falls slowly until, at the end of the tenth mile, it has reached the values of 0·80 and 0·81.

For the experiments shown in Table V we have not got a complete series of observations taken at half-hourly intervals during the period of rest after the work, since in some instances only three such observations were made. We give, however, for the sake of comparison, an average figure for such observations as we did make during this 3-hour period, and this will serve to show that the after-effects of the work were of similar character to those that will now be given in greater detail.

In Table VI we give the average figures which were obtained during a period of 3 hours' rest subsequent to a walk of 10 miles at a pace of 4·5 miles per hour in five experiments on Courtice and four on Douglas. In each of these experiments the respiratory exchange was determined at half-hourly intervals beginning half an hour after the work stopped.

TABLE VI—INFLUENCE OF 10-MILE WALK AT 4·5 MILES PER HOUR ON THE RESTING RESPIRATORY EXCHANGE. AVERAGE RESULTS. SUBJECTS POST-ABSORPTIVE

| | Courtice | | | Douglas | | |
|---|---|---|---|---|---|---|
| Time | Respiratory exchange cc/min | | R.Q. | Respiratory exchange cc/min | | R.Q. |
| | $CO_2$ | $O_2$ | | $CO_2$ | $O_2$ | |
| Rest before walk.... | 178 | 218 | 0·82 | 182 | 232 | 0·78 |
| ½ hour after walk .. | 179 | 232 | 0·77 | 178 | 257 | 0·69 |
| 1 ,, ,, .. | 181 | 232 | 0·78 | 177 | 244 | 0·73 |
| 1½ ,, ,, .. | 177 | 229 | 0·77 | 173 | 239 | 0·73 |
| 2 ,, ,, .. | 181 | 232 | 0·78 | 175 | 243 | 0·72 |
| 2½ ,, ,, .. | 179 | 231 | 0·77 | 173 | 239 | 0·73 |
| 3 ,, ,, .. | 181 | 234 | 0·77 | 180 | 241 | 0·75 |

In this table the most striking thing is the reduction of the respiratory quotient below the initial resting figure. In the case of Courtice the quotient remains quite steady at a figure 0·05 lower than that shown in the period of rest before the work began. Douglas shows an abnormally low figure for the quotient determined at the end of the first half-hour after the work stopped, but during the next 2½ hours gives figures which do not show any definite trend but have an average value about 0·05 lower than the initial resting quotient. Apart from the transitory very low quotient shown by Douglas at the end of the first half-hour the change of quotient shown by the two subjects seems to be very similar in type if we take into account the fact that Courtice had a higher quotient than Douglas when resting before the exercise. Courtice's $CO_2$ production during rest after the work is the same as before the work, and the low quotient corresponds with an increase in the oxygen consumption. Douglas's $CO_2$ production is somewhat lower, and the oxygen consumption somewhat higher, in the final rest period than they were during the preliminary rest.

It would appear therefore that if we exclude the determination at the end of the first half-hour on Douglas the post-exercise respiratory quotient falls to a level much below that shown before the exercise began, and that this level remains practically unchanged for as long as 3 hours. Courtice's quotient during the post-exercise period invariably remained steady at a low figure, but Douglas's quotient occasionally showed a tendency to creep upwards in the last 2½ hours. An instance of this will be seen in fig. 2, but even in this case the final respiratory quotient is only 0·75 as compared with the pre-exercise figure of about 0·825.

In a number of experiments we made fewer determinations of the respiratory exchange than those on which Table VI is based. An average of the whole of our available data is given in Table VII, excluding determinations made on Douglas at the end of the first half-hour after stopping work. This will serve to give a general idea of the persistent change in the resting respiratory quotient which results from the exercise.

TABLE VII—AVERAGE RESPIRATORY EXCHANGE AT REST, BEFORE, AND AFTER A 10-MILE WALK AT 4·5 MILES PER HOUR. SUBJECTS POST-ABSORPTIVE

| | Respiratory exchange cc/min | | R.Q. |
|---|---|---|---|
| | $CO_2$ | $O_2$ | |
| Courtice: 23 experiments— | | | |
| Before exercise | 181 | 223 | 0·81 |
| After exercise | 181 | 237 | 0·76 |
| Douglas: 15 experiments— | | | |
| Before exercise | 182 | 229 | 0·79 |
| After exercise | 176 | 240 | 0·73 |

In any argument regarding the proportions of carbohydrate and fat being oxidized which is based on the respiratory quotient the influence of the simultaneous metabolism of protein on this quotient must be taken into account. Strictly speaking, the nitrogen output in the urine can only be taken as an index of protein metabolism if the urine is collected over a long period, *e.g.*, 24 hours. At the same time we know that the protein metabolism of the body is almost uninfluenced by muscular work. We did, however, make some determinations of the total nitrogen excretion in the urine during the course of our experiments in order to get some idea of the order of magnitude of the protein metabolism. Table VIII gives the average results of two experiments on each subject.

TABLE VIII—NITROGEN EXCRETION IN THE URINE. SUBJECTS POST-ABSORPTIVE. WALK OF 10 MILES AT 4·5 MILES PER HOUR

| | Courtice | | Douglas | |
|---|---|---|---|---|
| | Urine cc/hour | Nitrogen mg/hour | Urine cc/hour | Nitrogen mg/hour |
| Rest before walk .................. | 78 | 620 | 167 | 723 |
| During walk ..................... | 70 | 499 | 83 | 442 |
| Rest 1st hour after walk ............ | 44 | 481 | 39 | 451 |
| „ 2nd „ ............ | 35 | 434 | 48 | 569 |
| „ 3rd „ ............ | 42 | 465 | 30 | 433 |

The rate of production of urine is obviously high in the earlier part of the experiments, diuresis being particularly marked during the preliminary rest of Douglas. This is due to the fluid deliberately drunk beforehand. The rate of production of urine is considerably reduced after the walk, but it never falls to a very low figure. As we nearly always drank water at the end of the walk this must have compensated in some measure for the loss of water in the sweat during the exercise. The nitrogen output per hour is at its highest during the preliminary rest period: no doubt this is dependent on the diuresis. During the rest of the experiment there is no great variation in the hourly rate of nitrogen output.

The preliminary period during which the urine was collected lasted for 2 hours, the walk for 2¼ hours, and the subsequent rest for 3 hours. Taking this into account the average rate of nitrogen output for the whole experiment is 520 mg per hour for Courtice and 540 mg for Douglas, corresponding to a protein metabolism of about 3·3 gm per hour. Assuming in accordance with Loewi's calculation as given by Lusk (1928) that the oxidation of 1 gm of protein in the body is accompanied by the production of 774 cc $CO_2$ and the consumption of 966 cc $O_2$, the oxidation of 3·3 gm of protein per hour would imply an output of 43 cc $CO_2$ and consumption of 53 cc $O_2$ per minute. In Table IX we show how the average respiratory quotients shown in Table VII will be affected if allowance is made for a protein metabolism at the above rate.

TABLE IX—AVERAGE OBSERVED R.Q. AND CALCULATED NON-PROTEIN R.Q.

| Subject | Rest before exercise | | Rest after exercise | |
|---|---|---|---|---|
| | Observed R.Q. | Non-protein R.Q. | Observed R.Q. | Non-protein R.Q. |
| Courtice | 0·81 | 0·81 | 0·76 | 0·75 |
| Douglas | 0·79 | 0·79 | 0·73 | 0·71 |

If it is assumed that the lower nitrogen output during and after the walk gives a fairer representation of the actual protein metabolism at the time the alteration in the respiratory quotients will be even less. In either case the non-protein respiratory quotient only differs sensibly from the observed quotient when the quotient is very low.

It will be clear that any discussion of the metabolic changes during the work must involve a consideration of the period of rest after the work is over, since any considerable alteration in comparison with what we observe during the initial resting period must be largely dependent on what has happened during the work. The figures given in Table IV indicate that there is no material alteration in the resting respiratory exchange and quotient over a period of 3 hours so long as the rest is uninterrupted by muscular exercise.

It has been shown in Table V that the respiratory quotient rises from 0·80 to 0·83 or 0·84 when the work is begun and then falls steadily though slowly to 0·80 or 0·81. Such a change of the respiratory quotient looks at first sight trifling. If we assume, however, that the observed quotient during the work is solely determined by the relative proportions of carbohydrate and fat being oxidized at the time, the part played by protein metabolism being negligible during the work, these proportions can be calculated by means of Zuntz's table (as given by Douglas and Priestley, 1924). Thus in Courtice's case 0·814 gm of carbohydrate and 0·494 gm of fat appear to be oxidized per minute at the end of the first 2 miles' walk, and 0·597 gm of carbohydrate and 0·574 gm of fat at the end of 10 miles. Corresponding figures for Douglas are 0·981 gm of carbohydrate and 0·517 gm of fat at the end of the first 2 miles, and 0·774 gm of carbohydrate and 0·632 gm of fat at the end of 10 miles. Although the change in the quotient may be small, it does imply, by this method of reckoning, that the proportions of carbohydrate to fat metabolized are very substantially altered as the work continues, and that of the energy liberated during the work the proportion due to the oxidation of carbohydrate to that due to the oxidation of fat is in the ratio of 1 : 1·2 in the early period of the work, whereas this ratio is 1 : 2·0 approximately in the final stages of the work.

Taking average values for the whole period of work shown in Table V the total metabolism would be represented by the figures given in Table X. For Douglas it must be remembered that some of the figures on which this table is based are taken from experiments in which he walked at 4·75 miles per hour. Taking only experiments done at a pace of 4·5 miles per hour we found an average consumption by him of 98 gm carbohydrate, 74 gm fat, and 7 gm protein during the work, giving a total energy output of 1121 calories.

TABLE X—AVERAGE TOTAL METABOLISM DURING A 10-MILE WALK AT 4½ MILES PER HOUR. SUBJECTS POST-ABSORPTIVE

| Substance metabolized | Courtice | | Douglas | |
|---|---|---|---|---|
| | Gm | Calories | Gm | Calories |
| Carbohydrate | 92 | 376 | 109 | 447 |
| Fat | 69 | 644 | 76 | 707 |
| Protein | 7 | 29 | 7 | 29 |
| | | 1049 | | 1183 |

Such a calculation of the nature of the substances metabolized is, however, only valid if other factors which are liable to disturb the respiratory quotient can be excluded. Leaving aside for the moment the possible conversion of carbohydrate into fat or fat into carbohydrate, with storage of the product formed, there are a number of other factors which may affect the respiratory quotient, and the presence or absence of such factors can be best gauged not merely by confining our attention to the period of work but by taking into consideration the phenomena shown during subsequent rest. Under this heading we have to take into account the possibility of temporary over- or under-breathing whilst collecting the bag sample, and the influence of change of body temperature, persistent alterations in the alveolar $CO_2$ pressure or $CO_2$ combining power of the blood, possible accumulation of lactic acid in the blood, and failure to complete the terminal stages of fat metabolism with the consequent accumulation of ketone bodies.

*Over- and under-breathing*—We do not believe that this factor was operative in our experiments. We were both well used to breathing through valves, and we always breathed through them for 5 minutes before starting to collect a bag sample of expired air so as to give time for any initial disturbance of the breathing to pass off.

*Body temperature*—During the walk the rectal temperature had risen 2° F, or rather more, by the time that the first circuit of the large track had been completed. A further rise of about 1° took place during the second circuit, and thereafter the rectal temperature remained steady. Typical observations on the behaviour of the rectal temperature after stopping the exercise are given in Table XI. It will be seen that Douglas's temperature just after stopping the work is nearly 1° above Courtice's. After half an hour's rest Courtice's temperature has fallen to a level which then remains steady for the rest of the experiment. Douglas's temperature on the other hand takes at least an hour to reach a steady level, and that level is well below that attained by Courtice.

TABLE XI—EFFECT OF A 10-MILE WALK AT 4·5 MILES PER HOUR ON THE RECTAL TEMPERATURE

| | Rectal temp. °F | | Rectal temp. °F |
|---|---|---|---|
| *Courtice* | | | |
| Before walk | 98·4 | Before walk | — |
| After stopping walk— | | After stopping walk— | |
| 2 min. | 101·0 | 4 min. | 101·1 |
| 7 ,, | 100·2 | 36 ,, | 98·2 |
| 12 ,, | 99·4 | 90 ,, | 98·3 |
| 17 ,, | 99·2 | 130 ,, | 98·2 |
| 22 ,, | 99·0 | 170 ,, | 98·3 |
| 27 ,, | 98·9 | 210 ,, | 98·4 |
| 32 ,, | 98·6 | | |
| 37 ,, | 98·5 | | |
| 42 ,, | 98·5 | | |
| 47 ,, | 98·5 | | |
| 52 ,, | 98·5 | | |
| 57 ,, | 98·6 | | |
| *Douglas* | | | |
| Before walk | 98·6 | Before walk | 98·4 |
| After stopping walk— | | After stopping walk— | |
| 7 min. | 101·8 | 5 min. | 101·7 |
| 51 ,, | 98·4 | 54 ,, | 98·1 |
| 77 ,, | 97·9 | 85 ,, | 97·7 |
| 113 ,, | 97·7 | 114 ,, | 97·6 |
| 138 ,, | 97·5 | 145 ,, | 97·6 |
| 177 ,, | 97·6 | 174 ,, | 97·6 |
| 202 ,, | 97·6 | 200 ,, | 97·7 |

Rise of body temperature increases the activity of the respiratory centre, and will therefore tend to cause over-ventilation and an increase of respiratory quotient whilst it is taking place; when the body temperature is falling again a retention of $CO_2$, with consequent lowering of the respiratory quotient, must occur. But the temperature which affects the respiratory centre cannot be in our experiments the same as the rectal temperature; it must be a good deal lower since in the heart warm blood coming back from the active muscles will get mixed with cool blood returning from the skin. We have no means of measuring the effective temperature of the blood reaching the brain, but we did take some readings of the mouth temperature as a possible index of this. Owing to the excessive cooling of the mouth by the hyperpnoea during the muscular exercise, and for some time afterwards, we felt that it was no use making the first measurement of mouth temperature until we had been at rest for 20 minutes after stopping the work. A comparison of the rectal and mouth temperatures in the case of Douglas is shown in fig. 3. It looks from this as though the effective temperature of the blood reaching the brain varied in the same sense as the rectal temperature though, of course, to nothing like the same degree.

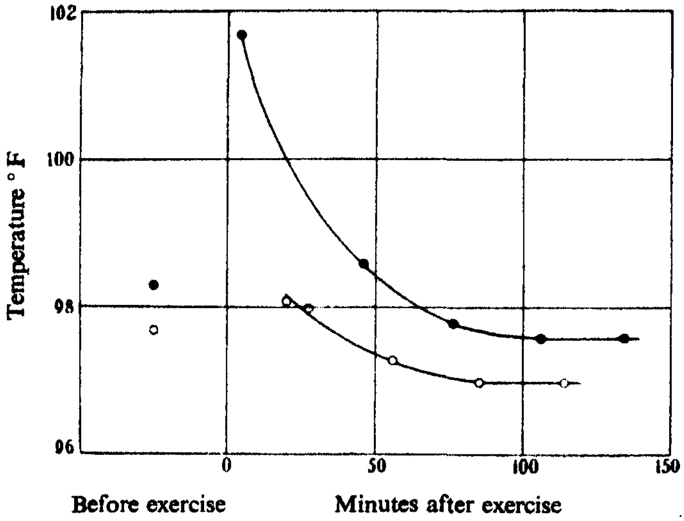


Fig. 3—Effect on rectal and mouth temperatures of a 10-mile walk at 4·5 miles per hour. ● rectal temperature; ○ mouth temperature.

Any influence which alteration of the body temperature may have on the respiratory quotient is really limited to the period when the temperature is changing. When once the temperature has become stable the respiration will reach an equilibrium in correspondence and the respiratory quotient will cease to be affected by the temperature. One would expect therefore that in our experiments if change of body temperature has any influence on the respiratory quotient such an effect will be limited to about the first half-hour of exercise when the body temperature is actually rising, and to about the first half-hour after the exercise stops in Courtice, and the first hour in Douglas, when the body temperature is falling. The tendency in the first instance will be to raise the respiratory quotient, in the second instance to lower it.

*Alveolar $CO_2$ pressure*—We have limited ourselves to observations made before beginning the exercise and at intervals after resuming rest. Observations made during the work are subject to some degree of error owing to the high rate at which $CO_2$ is being evolved at that time. Table XII gives the results of two typical experiments in which each subject walked for 10 miles at 4·5 miles per hour.

TABLE XII—RESTING ALVEOLAR $CO_2$ PRESSURE BEFORE AND AFTER A 10-MILE WALK AT 4·5 MILES PER HOUR

| | Alveolar $CO_2$ pressure mm Hg | | Alveolar $CO_2$ pressure mm Hg |
|---|---|---|---|
| *Courtice* | | *Douglas* | |
| Before walk | 41·2 | Before walk | 39·4 |
| After stopping walk— | | After stopping walk— | |
| 5 min. | 41·7 | 5 min. | 38·7 |
| 15 ,, | 41·4 | 15 ,, | 38·5 |
| 25 ,, | 41·1 | 25 ,, | 38·5 |
| 51 ,, | 40·6 | 48 ,, | 38·9 |
| 106 ,, | 41·6 | 98 ,, | 38·7 |
| 156 ,, | 41·4 | 143 ,, | 38·6 |

From these figures it will be seen that the alveolar $CO_2$ pressure after the exercise remains steady at a level which is practically identical with that shown before the exercise began. With Douglas, if there is a reduction in the alveolar $CO_2$ pressure, it is less than 1 mm. This result was confirmed in a number of other experiments, but in a few experiments Douglas did exhibit a definite, though slight reduction of the alveolar $CO_2$ pressure as a result of the exercise, and this reduction persisted for several hours. Instances of this will be referred to later.

*$CO_2$ combining power of the blood*—Although the resting alveolar $CO_2$ pressure seemed to be either unaffected by the exercise, or at most affected to but a trifling extent, the $CO_2$ combining power of the blood was found to be definitely, though slightly, lower than the pre-exercise value, in a sample of blood taken a minute or two after stopping the exercise, and it subsequently continued to fall slowly so that the lowest values were found in the third hour of rest after the exercise.

*Lactic acid in the blood*—A reduction in the $CO_2$ combining power of the blood naturally raises the suspicion that it may be associated with an increase in blood lactic acid. Although Owles's experiments had rendered it extremely improbable that there could be any significant accumulation of lactic acid during the exercise in our experiments we felt that it was desirable to confirm this. As our main interest in this connexion lay in the period of rest after the exercise was over we took the first sample of blood 3 to 5 minutes after resuming rest, and two further samples at later stages. Table XIII gives average figures for lactic acid in the blood in two experiments on each subject.

TABLE XIII—BLOOD LACTIC ACID DURING REST BEFORE AND AFTER A 10-MILE WALK AT 4·5 MILES PER HOUR

| *Courtice* | Mg lactic acid per 100 cc blood | *Douglas* | Mg lactic acid per 100 cc blood |
|---|---|---|---|
| Before walk ............ | 8·1 | Before walk ............ | 9·6 |
| 5 min. after walk ...... | 10·3 | 3 min. after walk ...... | 11·4 |
| 120 ,, ...... | 9·4 | 48 ,, ...... | 7·9 |
| 240 ,, ...... | 8·8 | 118 ,, ...... | 8·9 |

In blood samples collected from a vein in the arm 5 minutes after stopping the walk the lactic acid was about 2 mg higher per 100 cc of blood than when resting before the walk began. But this very small increase soon disappeared, and in determinations made at intervals from 45 minutes to 4 hours after the exercise was over values identical with the pre-exercise resting figure were shown in each subject. These observations are therefore in agreement with those made by Owles, and show that the type of work which we employed was insufficient to cause more than the most trivial accumulation of excess lactic acid in the blood. This fact is further corroborated by the absence of appreciable effect on the alveolar $CO_2$ pressure during the early stages of rest after the exercise.

After resuming rest the respiratory exchange falls very rapidly at first, but it takes about half an hour for Courtice, and an hour for Douglas, before it actually becomes steady. We cannot however take this to imply an oxygen debt in the sense in which this term is used by A. V. Hill. The oxidative removal of the trace of excess lactic acid which we found as a result of the exercise can be at most but a minor contributory cause of the excess oxygen consumption after the work is over. In any case the respiratory exchange cannot reach a steady resting level until the effects of the exercise on the heart and respiration have passed off, and there is one important factor which we must certainly not lose sight of in this connexion, viz., the changes of body temperature, since the metabolism of the body at large should continue to diminish until the body temperature falls to a constant figure, and this constant figure is not reached until half an hour to an hour have elapsed after resuming rest. If we suppose that the oxidative removal of traces of excess lactic acid and the falling body temperature result in a retention of $CO_2$ in the body, and are thus responsible, at least in some degree, for the maintenance of a low respiratory quotient in the early part of the post-exercise rest period, they cannot account for the persistent low respiratory quotient in the second and third hours of that period. Lactic acid accumulation cannot explain the fall in the $CO_2$ combining power of the blood, for although this fall is evident directly the work stops it steadily becomes greater during the whole 3 hours of rest long after the trace of excess lactic acid has disappeared from the blood.

*Ketone bodies in the blood and urine*—At an early stage of our experiments we found that urine secreted during the rest period after the exercise was over almost invariably gave a strong reaction with Rothera's nitro-prusside test for ketone bodies. With Courtice the reaction was negative in a few tests, and as a general rule it was less pronounced with him than with Douglas. In no instance did we ever get a positive Rothera reaction with urine secreted either during the preliminary rest or during the walk. This suggested to us the desirability of investigating the production of these ketone bodies in greater detail.

The failure to obtain a positive Rothera reaction in the urine secreted during the walk was not dependent on any decreased urinary output. The figures shown in Table XIV show that during the walk there was a free secretion of urine throughout which failed to give a Rothera reaction, although this reaction was positive in urine secreted during subsequent rest. Figures given later will show that during the 10-mile walk there was no increase in the ketone bodies in the blood. When ketonuria did develop after the exercise the Rothera reaction was always well marked

### TABLE XIV—DOUGLAS, POST-ABSORPTIVE. WALK AT 4·5 MILES PER HOUR

| | Urine cc/hour | Rothera reaction |
|---|---|---|
| 0 to 2·8 miles' walk | 185 | Negative |
| 2·8 to 6·2 miles' walk | 56 | Negative |
| 6·2 to 10·4 miles' walk | 57 | Negative |
| Rest during 3 hours after walk | 33 | Positive |

in the urine secreted during the first half-hour of rest, and though it might become more pronounced in later specimens of urine the ketosis evidently developed very rapidly after the cessation of exercise.

Courtice, as has been said, occasionally failed to show ketonuria, but if he somewhat restricted the carbohydrate in the diet taken on the day before, eating mainly fatty meat, eggs, etc., and diminishing the amount of bread taken, he always showed marked ketonuria after the exercise. Table XV shows the results of two experiments made on him: in A he had had his normal diet on the previous day whilst in B the carbohydrate in the diet had been restricted. Ketonuria occurred in each case after exercise, but to a far greater extent in experiment B. The Rothera reaction was strongly positive in the latter, though it was but faintly shown in experiment A.

### TABLE XV—COURTICE, POST-ABSORPTIVE. WALK OF 9·8 MILES AT 4·5 MILES PER HOUR

*Experiment A*—Normal diet on previous day. Post-exercise R.Q. 0·77. *Experiment B*—Carbohydrate in diet on previous day slightly restricted. Post-exercise R.Q. 0·74.

| *Experiment A* | Ketone bodies in urine mg/hr | *Experiment B* | Ketone bodies in urine mg/hr |
|---|---|---|---|
| Rest before walk | 2·9 | Rest before walk | — |
| During walk | 2·3 | During walk | 3·8 |
| 0 to 2 hours after walk | 9·7 | 0 to 1½ hours after walk | 21·4 |
| 2 to 3 „ | 8·7 | 1½ to 3 „ | 35·3 |
| 3 to 4 „ | 7·9 | 3 to 4 „ | 41·8 |

*Note.*—" Ketone bodies " are expressed throughout as β-hydroxybutyric acid.

Table XVI gives the results obtained on the same subject in which the course of events after the exercise was over was followed for 9½ hours instead of the usual 3. Several interesting points are shown in this table. The respiratory exchange during the whole of this long period of rest remains very steady. The $CO_2$ production is on the average 10 cc and the $O_2$ consumption 28 cc above the initial resting figures, and the respiratory quotient is 0·75 in contrast with the pre-exercise figure of 0·80. Mere prolongation of the resting period beyond the customary 3 hours does not therefore result in the respiratory quotient returning to its original value. In this experiment the alveolar $CO_2$ pressure shows a slight though progressive decrease during the post-exercise period. Although

TABLE XVI—COURTICE, POST-ABSORPTIVE. WALK OF 10 MILES AT 4·5 MILES PER HOUR

| | Respiratory exchange cc/min. | | R.Q. | Alveolar $CO_2$ pressure mm Hg | Urine cc/hour | Urine ketone bodies mg/hr |
|---|---|---|---|---|---|---|
| | $CO_2$ | $O_2$ | | | | |
| Preliminary rest | 173 | 215 | 0·80 | 41·6 | | |
| | 171 | 218 | 0·79 | 41·6 | 40 | 10 |
| During walk .. | — | — | — | — | 37 | 11 |
| Rest after walk for— | | | | | | |
| h. min. | | | | | | |
| 0 12 | — | — | — | 42·5 | 29 | 47 |
| 0 35 | 188 | 249 | 0·75 | — | | |
| 0 52 | — | — | — | 41·5 | | |
| 1 20 | 190 | 250 | 0·76 | — | | |
| 2 5 | 179 | 249 | 0·72 | — | 24 | 131 |
| 2 20 | — | — | — | 40·7 | | |
| 2 50 | 177 | 237 | 0·75 | — | | |
| 3 35 | 180 | 239 | 0·75 | — | 24 | 68 |
| 4 20 | 181 | 237 | 0·76 | — | | |
| 4 34 | — | — | — | 40·4 | 55 | 90 |
| 5 5 | 196 | 249 | 0·78 | — | | |
| 5 50 | 189 | 255 | 0·74 | — | | |
| 6 35 | 181 | 245 | 0·74 | — | 58 | 102 |
| 6 49 | — | — | — | 39·0 | | |
| 7 20 | 184 | 247 | 0·75 | — | | |
| 8 5 | 181 | 250 | 0·72 | — | 48 | 117 |
| 8 50 | 182 | 250 | 0·73 | — | | |
| 9 4 | — | — | — | 39·3 | 29 | 79 |
| 9 35 | 175 | 234 | 0·75 | — | | |

quantitative analysis showed that the urine secreted during the preliminary rest and during the walk contained ketone bodies equivalent to the excretion of about 10 mg of β-hydroxybutyric acid per hour the Rothera test was negative: possibly this result is to be explained by the nature of the ketone bodies excreted, since acetone and aceto-acetic acid give the Rothera test while β-hydroxybutyric acid does not. During the subsequent rest period when the Rothera test was strongly positive the rate of excretion of ketone bodies, expressed as β-hydroxybutyric acid, is strikingly and persistently increased.

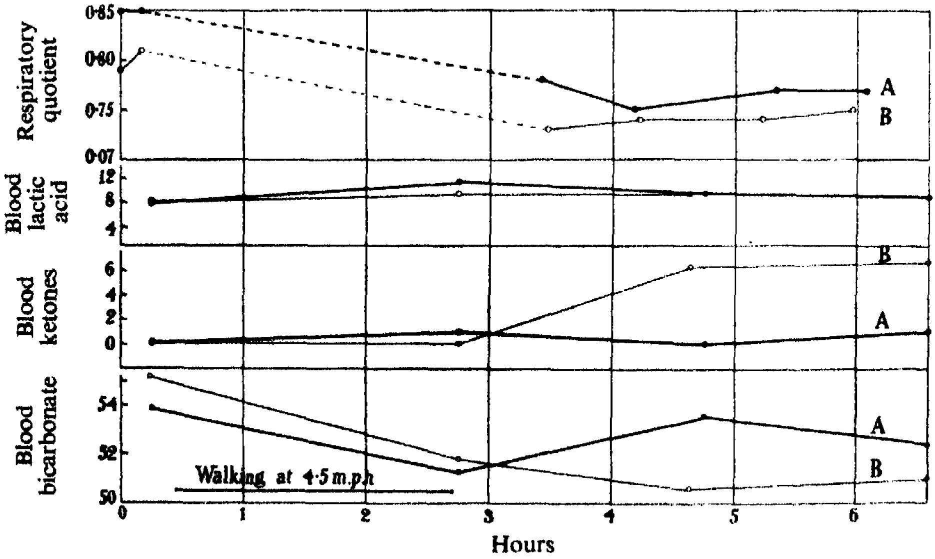


FIG. 4—Courtice. Blood lactic acid in mg %. Blood ketones expressed as mg β-hydroxybutyric acid %. Blood bicarbonate = $CO_2$ combining power of blood in volumes %. A, normal diet on previous day; B, carbohydrate in diet on previous day slightly restricted.

Owing to the large variations in the volume and concentration of the urine excreted during an experiment the determination of the ketone bodies excreted in the urine will not give a good indication of the concentration of the ketone bodies in the tissues. We have therefore determined in addition the concentration of ketone bodies in the blood.

In fig. 4 we have plotted additional data obtained in two experiments on Courtice which have already been referred to in Table XV.

In both experiments the change in lactic acid concentration in the blood is insignificant, but in experiment B there is a definite rise of some 6 mg per 100 cc in the ketone bodies of the blood and a fall of about

4 volumes per cent in the $CO_2$ combining power of the blood in the post-exercise period, whereas in experiment A the change of ketone bodies in the blood is inappreciable and the reduction of $CO_2$ combining power certainly much less. This suggests that the progressive reduction in the $CO_2$ combining power of the blood which was so often found in our experiments is connected with the appearance of the ketone bodies.

It might be thought that the readiness with which ketonuria is produced is possibly dependent not so much on the duration of the work as on the rate at which the work is done. In some experiments we reduced the rate of walking from 4·5 to 3·5 miles per hour. This reduced the oxygen consumption per minute from about 1800 cc to 1200 in Douglas and from about 1650 cc to 1150 cc in Courtice. In order that the same total amount of oxygen should be used during the walk at the slower rate as at the faster allowance had to be made for the increased efficiency per kg m horizontal work at the slower pace. To make up for this we walked 12 miles instead of 10, the walk taking therefore 3½ hours instead of 2¼. An example of one of these experiments on Douglas is given in Table XVII. It will be noticed that during the walk at the slower pace the respiratory quotient does not rise above the initial resting level, and that there is only a slight progressive fall as the exercise continues. This was confirmed in other experiments made in the same way. The respiratory quotient after the work is over behaves in very much the same way as in

TABLE XVII—DOUGLAS, POST-ABSORPTIVE. WALK OF 12 MILES AT 3·5 MILES PER HOUR

| | Respiratory exchange cc/min | | R.Q. |
|---|---|---|---|
| | $CO_2$ | $O_2$ | |
| Rest before walk | 197 | 246 | 0·80 |
| ,, | 191 | 235 | 0·81 |
| End of 2 miles' walk | 1012 | 1277 | 0·79 |
| ,, 4 ,, | 924 | 1199 | 0·77 |
| ,, 6 ,, | 941 | 1182 | 0·80 |
| ,, 8 ,, | 984 | 1263 | 0·78 |
| ,, 10 ,, | 969 | 1244 | 0·78 |
| ,, 12 ,, | 928 | 1209 | 0·77 |
| Rest ½ hour after walk | 164 | 234 | 0·70 |
| ,, 1 ,, | 178 | 237 | 0·75 |
| ,, 1½ ,, | 171 | 233 | 0·73 |
| ,, 2 ,, | 173 | 231 | 0·75 |
| ,, 2½ ,, | 176 | 232 | 0·76 |
| ,, 3 ,, | 178 | 235 | 0·76 |

experiments at the faster pace, save that the level finally reached is perhaps rather higher than in those experiments. The Rothera test was positive in urine secreted after exercise in this and other experiments of the same type. Even after a walk of 10 miles at 3·5 miles per hour the Rothera test was faintly positive, but it was much stronger when the distance was increased to 12 miles, though we thought that the ketonuria was even then rather less pronounced than when we walked at the faster pace. A factor which plays a large part in determining the ketonuria is evidently the absolute amount of work done irrespective of the rate at which it is done, and it looks therefore as though the main result of our experiments was to antedate the ketosis which develops during simple starvation.

The ketonuria resulting from exercise was easily abolished by ingesting sugar as is shown in Table XVIII in an experiment made on Courtice.

TABLE XVIII—COURTICE. EFFECT OF INGESTION OF SUCROSE ON POST-EXERCISE KETOSIS

| | Respiratory exchange cc/min | | R.Q. |
|---|---|---|---|
| | $CO_2$ | $O_2$ | |
| Rest before walk | 170 | 215 | 0·79 |
| ,, | 176 | 217 | 0·81 |
| Rest— | | | |
| 45 min. after walk | 170 | 232 | 0·73 |
| 90 ,, | 170 | 228 | 0·74 |
| 150 ,, | 184 | 248 | 0·74 |
| 195 ,, | 180 | 240 | 0·75 |
| 245 ,, | Ingested 100 gm sucrose | | |
| 35 min. after sucrose | 223 | 268 | 0·83 |
| 80 ,, | 248 | 276 | 0·90 |
| 125 ,, | 204 | 236 | 0·86 |
| 170 ,, | 208 | 250 | 0·83 |

| | Blood ketone bodies mg % | | Urine ketone bodies mg/hr |
|---|---|---|---|
| Before walk | 0·0 | Before walk | 9·5 |
| | | During walk | 3·8 |
| 4 min. after walk | 0·0 | 8–103 min. after walk | 21·4 |
| 116 ,, | 6·3 | 103–166 ,, | 35·3 |
| 236 ,, | 6·7 | 166–243 ,, | 41·7 |
| | | 0–2 hours after sucrose | 11·8 |
| 120 min. after sucrose | 0·0 | 2–4 ,, | 0·0 |

During 4 hours' rest after a 10-mile walk at the rate of 4·5 miles per hour there was a considerable increase in the ketone bodies in the blood and in the amount of these bodies excreted in the urine. At the end of this time 110 gm of sucrose were ingested with the result that there was a considerable rise in the respiratory quotient and a rapid disappearance of the ketone bodies.

### THE INFLUENCE OF CARBOHYDRATE FOOD TAKEN BEFORE THE MUSCULAR EXERCISE

It has been pointed out in the preceding section that although Douglas invariably showed a ketosis as a result of the exercise, Courtice in some experiments did not, whilst his respiratory quotient during post-exercise rest was higher than Douglas's. It will also be seen from the average figures given in Table VII that Courtice tends to have a rather higher initial respiratory quotient during rest than Douglas. The diet taken by the two subjects on the days preceding these experiments was their natural one, and it seemed possible that the differences in quotient might depend either on the fact that Courtice naturally selects a diet richer in carbohydrate than Douglas or that he can store carbohydrate in available form more readily.

We therefore tried the effects of increasing considerably the carbohydrate in the diet taken on the day preceding the experiment. Courtice ate principally bread, biscuits, and jam and took a good deal of sucrose in addition: Douglas ate porridge with sugar, bread, jam, and spaghetti. The actual experiments were of course made with the subjects in the post-absorptive state.

The results of two typical experiments are shown in Tables XIX and XX.

The respiratory quotient during preliminary rest, during the walk, and during the subsequent period of rest is higher than in the experiments previously described when the subjects had been taking their normal diet on the previous day. The general picture is much the same save that when the carbohydrate diet had been taken on the previous day the respiratory quotient runs throughout at a higher level. Fig. 5 shows the contrast between the respiratory quotients of the two subjects under different circumstances.

The upper pair of curves shows the changes of the respiratory quotient in Courtice (A) and in Douglas (B) when a normal diet had been taken on the previous day in each case. The curves are drawn from the average figures in all those experiments in which we had a full series of deter-

TABLE XIX—COURTICE, POST-ABSORPTIVE. HIGH CARBOHYDRATE DIET ON PRECEDING DAY. WALK OF 10 MILES AT 4·5 MILES PER HOUR

| | Respiratory exchange cc/min | | R.Q. |
|---|---|---|---|
| | $CO_2$ | $O_2$ | |
| Rest before walk | 195 | 210 | 0·93 |
| ,, | 191 | 203 | 0·94 |
| End of 2 miles' walk | 1309 | 1468 | 0·89 |
| ,, 4 ,, | 1365 | 1507 | 0·91 |
| ,, 6 ,, | 1355 | 1509 | 0·90 |
| ,, 8 ,, | 1325 | 1497 | 0·88 |
| ,, 10 ,, | 1296 | 1485 | 0·87 |
| Rest ½ hour after walk | 180 | 217 | 0·83 |
| ,, 1¼ ,, | 181 | 217 | 0·83 |
| ,, 1¾ ,, | 180 | 224 | 0·80 |

TABLE XX—DOUGLAS, POST-ABSORPTIVE. HIGH CARBOHYDRATE DIET ON PRECEDING DAY. WALK OF 10 MILES AT 4·5 MILES PER HOUR

| | Respiratory exchange cc/min | | R.Q. |
|---|---|---|---|
| | $CO_2$ | $O_2$ | |
| Rest before walk | 185 | 220 | 0·84 |
| ,, | 185 | 213 | 0·87 |
| End of 4·2 miles' walk | 1415 | 1648 | 0·86 |
| ,, 7·9 ,, | 1446 | 1694 | 0·85 |
| Rest ½ hour after walk | 176 | 236 | 0·74 |
| ,, 1 ,, | 172 | 221 | 0·78 |
| ,, 1½ ,, | 169 | 218 | 0·78 |
| ,, 2 ,, | 172 | 219 | 0·78 |
| ,, 2½ ,, | 167 | 216 | 0·77 |
| ,, 3 ,, | 164 | 212 | 0·77 |

minations during the work as well as after it. In the middle pair of curves the same curve (A) for Courtice is contrasted with curve C which is based on the average figures shown when he had restricted to some degree the carbohydrate taken on the previous day. It will be seen that this restriction of carbohydrate has the result that his post-exercise respiratory quotient is practically identical with that of Douglas (curve B) who was taking his normal diet. In the lower pair of curves, curve B for Douglas is contrasted with curve D which gives the result of an experiment in which he had taken a greatly increased amount of carbohydrate on the previous day.

In the three experiments made on Douglas and the three on Courtice in which an increased amount of carbohydrate was taken on the previous day the urine collected at intervals during the post-exercise period failed to give a positive Rothera reaction. Blood samples taken from a vein in the arm showed that there was no persistent diminution of the $CO_2$ combining power such as has been described in the previous section. This will be clear from the results given in Table XXI which were obtained in two experiments on each subject, in one of which a normal diet had been taken on the day before and in the other a carbohydrate rich diet. On the latter diet the $CO_2$ combining power of the blood of each subject, especially of Douglas, shows a slight fall in samples taken just after the exercise, but in further samples taken at the end of the first and second hours' rest the original $CO_2$ combining power has been regained, although

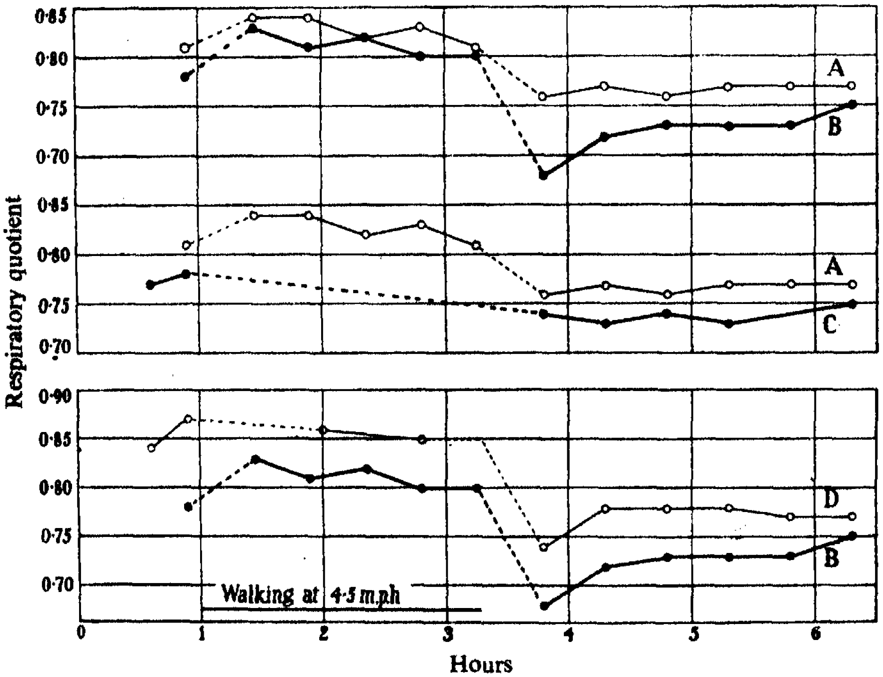


Fig. 5—Subjects post-absorptive. A, Courtice, normal diet previous day; B, Douglas, normal diet previous day; C, Courtice, carbohydrate somewhat restricted on previous day; D, Douglas, high carbohydrate diet on previous day.

TABLE XXI—SUBJECTS POST-ABSORPTIVE. 10-MILE WALK AT 4·5 MILES PER HOUR. EFFECT OF EXERCISE ON THE $CO_2$ COMBINING POWER OF THE BLOOD. NORMAL DIET TAKEN ON PREVIOUS DAY IN ONE CASE, A HIGH CARBOHYDRATE DIET IN THE OTHER

| | Normal diet: ketosis present | | High carbohydrate diet: no ketosis | |
|---|---|---|---|---|
| | $CO_2$ pressure mm Hg | $CO_2$ vols. per 100 cc blood | $CO_2$ pressure mm Hg | $CO_2$ vols. per 100 cc blood |
| *Douglas* | | | | |
| Rest before walk | 41·5 | 53·4 | 40·8 | 53·4 |
| Rest 5 min. after walk | 41·9 | 51·4 | 41·4 | 51·9 |
| „ 53 „ | 42·2 | 50·8 | 41·5 | 52·6 |
| „ 130 „ | 42·1 | 50·3 | 41·7 | 53·3 |
| „ 166 „ | 41·7 | 50·5 | | |
| *Courtice* | | | | |
| Rest before walk | 41·4 | 53·2 | 41·8 | 53·7 |
| Rest 3 min. after walk | 41·3 | 52·5 | 41·8 | 52·9 |
| „ 50 „ | 41·4 | 52·8 | 42·1 | 53·9 |
| „ 120 „ | 41·3 | 51·6 | 42·0 | 54·0 |

in the experiments in which a normal diet had been taken beforehand the $CO_2$ combining power continued, if anything, to drop further. Although the slight fall in the $CO_2$ combining power just after the exercise may be dependent on the trifling accumulation of excess lactic acid in the blood at this time, it is clear that the further and continued fall must be due to the accumulation of ketone bodies, since it only occurs in those experiments in which there is evidence of ketosis.

Taking our experiments as a whole it can be said that ketonuria was always present when the resting respiratory quotient was 0·76 or lower. With a respiratory quotient of 0·77 ketonuria sometimes occurred, and even with a respiratory quotient of 0·78 we occasionally got a faint positive result with the Rothera test.

Although post-exercise ketonuria on the day of the experiment could apparently be readily prevented by taking plentiful carbohydrate on the day before, the case was different when the subject deliberately took food shortly before the start of the experiment. In these experiments he was, of course, no longer in the post-absorptive condition. Table XXII gives the result of one experiment on each subject in which he had had his normal breakfast (eggs or cutlets and bacon, toast, jam, and tea) before beginning the experiment. Courtice sat down to rest in the laboratory 40 minutes after breakfast and Douglas 20 minutes, so that the first respiratory exchange determinations at rest were made roughly 1 hour after breakfast. The respiratory exchange during the initial rest is very much greater than in the post-absorptive experiments, although the respiratory quotient is only slightly above the average for the latter experiments. With Douglas the respiratory quotient during the work is very much the same as that shown in the post-absorptive experiments, but with Courtice it is definitely higher. After the exercise is over Douglas's respiratory quotient falls to the surprisingly low level of 0·71, and it remains steady at this figure until the end of the third hour of rest. This was, in fact, the lowest persistent respiratory quotient which occurred in the whole course of our experiments, and it was accompanied, as would be expected, by an intense Rothera reaction in the urine. On the other hand Courtice's respiratory quotient in the post-exercise period only varies between 0·77 and 0·75, figures similar to those shown in the ordinary post-absorptive experiments. His urine, too, at this time gave a

TABLE XXII—EFFECT OF A 10-MILE WALK AT 4·5 MILES PER HOUR AFTER A NORMAL BREAKFAST

| | Douglas | | | Courtice | | |
|---|---|---|---|---|---|---|
| | Respiratory exchange cc/min | | R.Q. | Respiratory exchange cc/min | | R.Q. |
| | $CO_2$ | $O_2$ | | $CO_2$ | $O_2$ | |
| Rest before walk .. | 237 | 295 | 0·80 | 242 | 290 | 0·83 |
| ,, .. | 242 | 290 | 0·84 | 247 | 297 | 0·83 |
| End of— | | | | | | |
| 2 miles' walk.... | 1528 | 1799 | 0·85 | 1404 | 1602 | 0·88 |
| 4 ,, .... | 1495 | 1818 | 0·82 | 1409 | 1588 | 0·89 |
| 6 ,, .... | 1439 | 1789 | 0·80 | 1404 | 1612 | 0·87 |
| 8 ,, .... | 1452 | 1811 | 0·80 | 1409 | 1629 | 0·86 |
| 10 ,, .... | — | — | — | 1386 | 1633 | 0·85 |
| Rest— | | | | | | |
| ½ hour after walk | 185 | 264 | 0·70 | — | — | — |
| 1 ,, | 181 | 256 | 0·71 | 200 | 260 | 0·77 |
| 2 ,, | 177 | 248 | 0·72 | 196 | 254 | 0·77 |
| 2½ ,, | 171 | 242 | 0·71 | — | — | — |
| 3 ,, | 170 | 241 | 0·71 | 205 | 273 | 0·75 |
| 4 ,, | — | — | — | 204 | 272 | 0·75 |

positive Rothera reaction. Even when Douglas took 60 gm of sucrose with porridge for breakfast ketonuria still developed after the walk though not to a marked degree. During the walk the respiratory quotient was much higher than in the usual post-absorptive experiments. It is however, unnecessary to refer in detail to these experiments since the facts are just as clearly shown when the breakfast is practically limited to sugar.

In this case we took 50 gm of either glucose or fructose (B.D.H. laevulose) and from 20 to 40 gm of bread, which would contain from 10 to 20 gm of starch. The sugar was dissolved as a rule in tea, but sometimes in lemonade, and the bread was eaten in the hopes that it might help to slow down the rate of absorption of sugar from the gut. This carbohydrate meal was taken from 20 to 30 minutes before sitting down for the preliminary rest, and the walk was begun from 80 to 110 minutes after the meal had been taken, by which time the bulk of the sugar should have been absorbed.

Tables XXIII and XXIV give the average results of six experiments on each subject, in three of which glucose had been ingested and in the other three fructose. The individual experiments in any one of these groups of three gave almost identical results.

TABLE XXIII—COURTICE. EFFECT OF WALKING 10 MILES AT 4·5 MILES PER HOUR AFTER INGESTION OF EITHER GLUCOSE OR FRUCTOSE

| | After glucose | | | After fructose | | |
|---|---|---|---|---|---|---|
| | Respiratory exchange cc/min | | R.Q. | Respiratory exchange cc/min | | R.Q. |
| | $CO_2$ | $O_2$ | | $CO_2$ | $O_2$ | |
| Rest before walk .. | 241 | 264 | 0·91 | 252 | 259 | 0·97 |
| ,, .. | 235 | 257 | 0·91 | 250 | 262 | 0·95 |
| End of— | | | | | | |
| 2 miles' walk .... | 1515 | 1630 | 0·93 | 1448 | 1629 | 0·89 |
| 4 ,, .... | 1414 | 1615 | 0·88 | 1410 | 1628 | 0·87 |
| 6 ,, .... | 1372 | 1620 | 0·85 | 1421 | 1660 | 0·86 |
| 8 ,, .... | 1339 | 1626 | 0·82 | 1372 | 1642 | 0·84 |
| 10 ,, .... | 1360 | 1690 | 0·80 | 1379 | 1700 | 0·81 |
| Rest— | | | | | | |
| ½ hour after walk | 179 | 242 | 0·74 | 180 | 235 | 0·77 |
| 1 ,, | 187 | 245 | 0·76 | 180 | 236 | 0·77 |
| 1½ ,, | 177 | 236 | 0·75 | 182 | 238 | 0·77 |
| 2 ,, | 181 | 238 | 0·76 | 186 | 237 | 0·78 |
| 2½ ,, | 181 | 239 | 0·76 | 179 | 232 | 0·77 |

The respiratory exchange during the initial rest period is considerably higher than in the post-absorptive experiments, but during the walk and the post-exercise rest period the oxygen consumption is practically the same as in the latter experiments. The respiratory quotient during the initial rest is about 0·91 in the glucose experiments and 0·95 in the fructose experiments. After walking 2 miles the respiratory quotient has risen to about 0·94 in the glucose experiments but has fallen to about 0·90 in the fructose ones. Thereafter the respiratory quotient falls steadily, the figures being nearly identical for each sugar. At the end of the tenth mile Courtice's quotient is practically the same as in the post-absorptive experiments, while Douglas's is a little higher, *cf.* Table V. In the post-exercise rest period both respiratory exchange and quotient quickly fall to a steady level. Both subjects have practically the same quotient during this period. This is the same as that shown by Courtice in the ordinary post-absorptive experiments, but for Douglas is definitely higher than in the latter, *cf.* Tables VI and VII. The respiratory quotients in these experiments have been plotted in fig. 6. The results are clearly very much the same no matter whether glucose or fructose has been

TABLE XXIV—DOUGLAS. EFFECT OF WALKING 10 MILES AT 4·5 MILES PER HOUR AFTER INGESTION OF EITHER GLUCOSE OR FRUCTOSE

| | After glucose | | | After fructose | | |
|---|---|---|---|---|---|---|
| | Respiratory exchange cc/min | | R.Q. | Respiratory exchange cc/min | | R.Q. |
| | $CO_2$ | $O_2$ | | $CO_2$ | $O_2$ | |
| Rest before walk .. | 258 | 291 | 0·89 | 268 | 281 | 0·95 |
| „ .... | 258 | 283 | 0·91 | 263 | 279 | 0·95 |
| End of— | | | | | | |
| 2 miles' walk .... | 1635 | 1722 | 0·95 | 1622 | 1790 | 0·91 |
| 4 „ .... | 1540 | 1744 | 0·88 | 1570 | 1776 | 0·88 |
| 6 „ .... | 1560 | 1784 | 0·87 | 1520 | 1759 | 0·86 |
| 8 „ .... | 1534 | 1816 | 0·85 | 1592 | 1855 | 0·86 |
| 10 „ .... | 1522 | 1837 | 0·83 | 1544 | 1832 | 0·83 |
| Rest— | | | | | | |
| ½ hour after walk | 179 | 255 | 0·70 | 186 | 252 | 0·74 |
| 1 „ | 182 | 244 | 0·75 | 182 | 242 | 0·74 |
| 1½ „ | 182 | 238 | 0·76 | 179 | 233 | 0·77 |
| 2 „ | 177 | 233 | 0·76 | 180 | 232 | 0·78 |
| 2½ „ | 178 | 238 | 0·75 | 178 | 230 | 0·77 |

taken beforehand, but with fructose the respiratory quotient in the post-exercise period tends to be the higher.

In all of these experiments a positive Rothera reaction was given in the urine secreted during the post-exercise rest period, save in one experiment with glucose on Douglas, and in one experiment with fructose on each subject. We got the impression that the Rothera reaction was less marked in the fructose than in the glucose experiments.

The regularity with which ketosis resulted from the muscular work in our normal post-absorptive experiments, and particularly in the experiments in which sugar was ingested shortly before beginning the work, surprised us, for a 10-mile walk cannot be regarded as excessive exercise even if one has had no breakfast before doing it. The ketosis which develops during starvation is well known, as is the fact that comparatively light work may cause some degree of ketosis in subjects who have been taking a diet in which the carbohydrate has been greatly reduced and the fat increased (*cf.* Gemmill, 1934), but our experiments were made under quite different nutritive conditions.

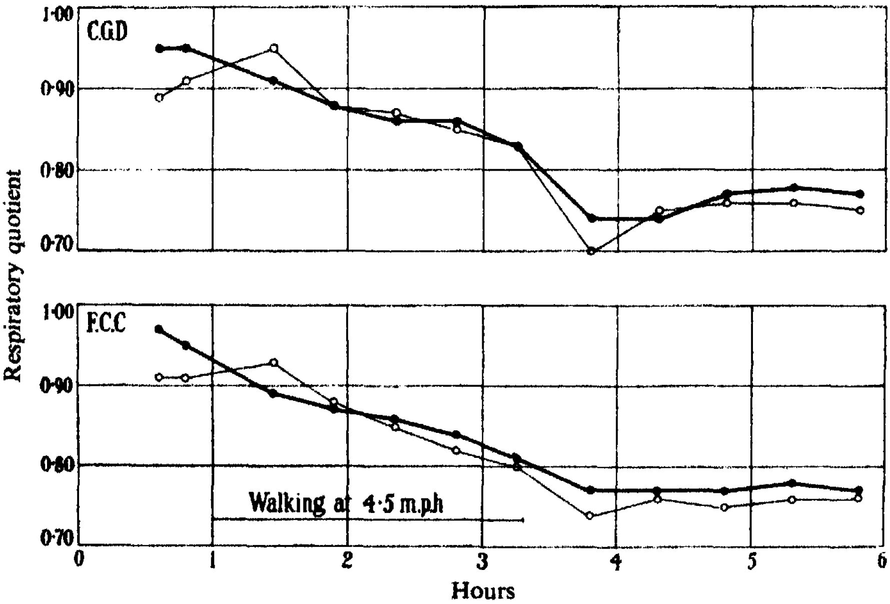


FIG. 6—Effect on the respiratory quotient of a 10-mile walk at 4·5 miles per hour after ingesting either glucose or fructose. ● Fructose; ○ glucose.

Since phosphate plays so essential a part in the chemical changes associated with muscular contraction and Embden, Grafe, and Schmitz

(1921) have claimed that the muscular efficiency during active exercise may be increased by the ingestion of phosphate, we made two experiments on Douglas in the post-absorptive state when he had had his normal diet on the day before to test the possible influence of phosphate. In the first of these the subject took 2·5 gm of $NaH_2PO_4$ at 11 p.m. on the previous evening and another 2·5 gm 2 hours before beginning the experiment. In the second he took 5 gm at 6.30 p.m. and 5 gm at 11 p.m. on the day before and another 5 gm 2 hours before beginning the experiment. Determinations of the alveolar $CO_2$ pressure showed that there was no appreciable acidosis, and we could detect no difference in either the respiratory exchange or the respiratory quotient from what was found in ordinary post-absorptive experiments. Douglas was unable to appreciate any change in his subjective sensations of fatigue after taking the phosphate.

### EFFECT OF REPETITION OF THE EXERCISE

Since the result of a 10-mile walk was liable to cause so marked and persistent a reduction of the respiratory quotient during subsequent rest it seemed to us desirable to repeat the walk when the subject had rested for some time after walking 10 miles.

All these experiments were made with the subject in the post-absorptive state after taking his normal diet on the previous day. After walking 10 miles at 4·5 miles per hour he sat down at rest for 2 hours and then walked another 6 miles at the same pace, finally sitting down at rest again for another 2 hours. The respiratory exchange was determined during the initial rest and three times during each of the work and subsequent rest periods. Blood for the determination of the ketone bodies and $CO_2$ combining power was taken during the initial rest and at the end of each period of work and subsequent rest. The alveolar $CO_2$ pressure was determined at intervals during the resting periods, and urine samples were taken for the quantitative estimation of the ketonuria.

Fig. 7 gives the average results obtained on Douglas in three experiments which were in close agreement.

It will be seen that the respiratory exchange and quotient follow quite the usual course during the first walk of 10 miles and the 2-hour period of rest following it. The respiratory quotient during the latter period remains steady at about 0·73 in contrast with the initial pre-exercise figure of 0·80. During the second walk of 6 miles the respiratory exchange mounts to about the same level as in the previous walk and the quotient rises to 0·78, but it does not attain the value of 0·81 shown towards the

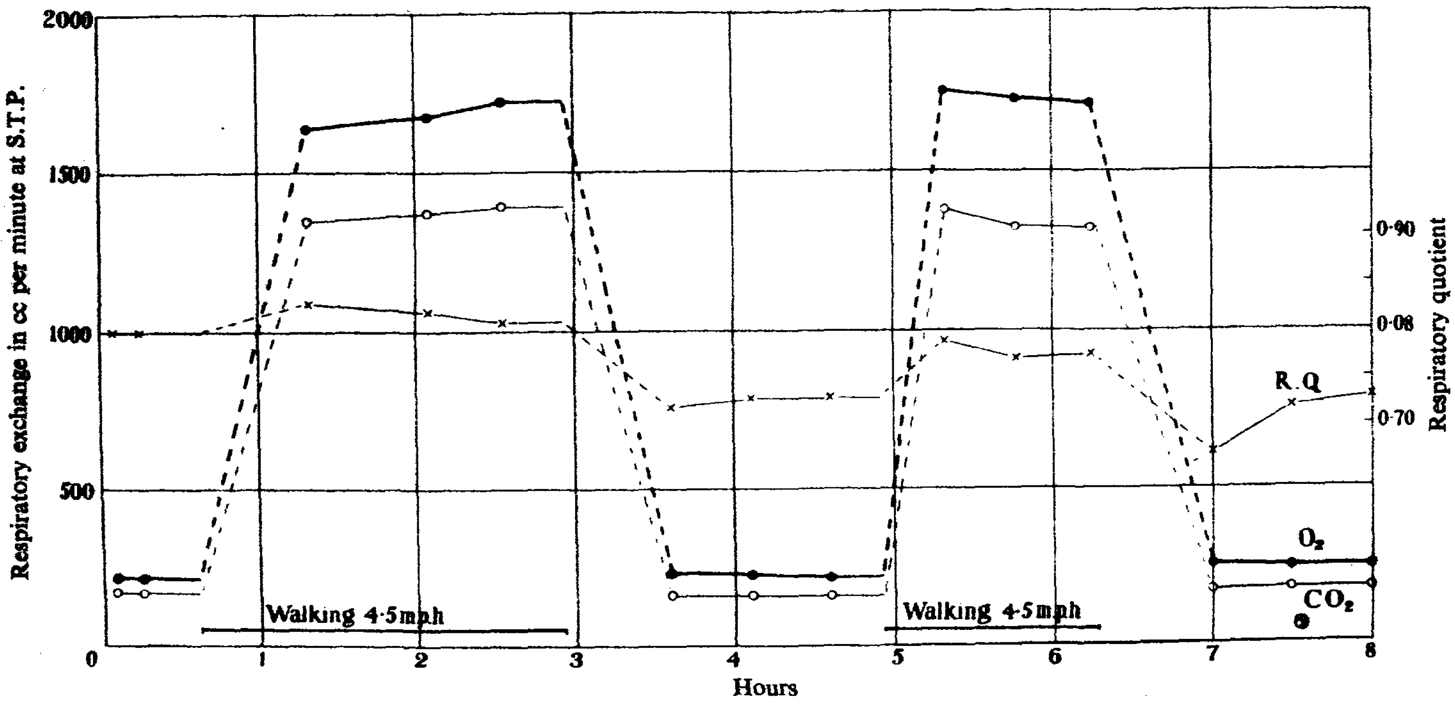


FIG. 7—Douglas, post-absorptive. Effect of walking 10 miles at 4·5 miles per hour followed by a further walk of 6 miles at the same pace after an interval of rest of 2 hours. × R.Q.; ● $O_2$; ○ $CO_2$.

end of the earlier walk. This raised quotient is, however, maintained steady during the second walk to fall markedly again during the final rest.

Blood and alveolar air analyses were only done in two of these experiments, and the results of these are given in fig. 8. The changes of respiratory quotient in these two experiments are practically identical with those shown in fig. 7. The concentration of ketone bodies in the blood is almost unaltered by the first period of exercise, rises slightly during rest, the rise is continued during the second walk and there is a further sharp increase in the final period of rest. The $CO_2$ combining power of the blood falls more or less steadily until the end of the second walk, and in the final period of rest seems to be decreasing more rapidly, as might be expected from the rapid increase of the ketone bodies at this time. The resting alveolar $CO_2$ pressure is slightly diminished as a result of the first walk, but it is difficult to say that any further change takes place after the second walk: in both periods of rest after the exercise the average figure for the alveolar $CO_2$ pressure is, however, only about 1·5 mm below the initial resting figure. It will be noticed that the fall of alveolar $CO_2$ pressure is disproportionally small compared with the reduction of the $CO_2$ combining power of the blood. If, therefore, the $p_H$ of the blood is calculated from the ratio of the free to the combined $CO_2$ there appears to be a fall, although as the breathing was perfectly quiet at the time the respiratory centre did not seem to be responding to any such change of hydrogen ion concentration. This apparent discrepancy between the calculated $p_H$ of the blood and the behaviour of the respiratory centre may be but another of those anomalous instances which have been discussed by Douglas and Havard (1932), but the matter needs further investigation. The results of the urine analyses are given in Table XXV. These show the increase in the rate of excretion of

TABLE XXV—DOUGLAS, POST-ABSORPTIVE. EFFECT OF DOUBLE WALK ON EXCRETION OF KETONE BODIES IN THE URINE

| | Urine cc/hour | Ketone bodies mg/hour |
|---|---|---|
| Rest before walk | 203 | 4·3 |
| During 1st walk of 10 miles | 40 | 2·7 |
| Rest 1st hour after walk | 31 | 9·5 |
| „ 2nd „ | 85 | 10·5 |
| During 2nd walk of 6 miles | 9 | 2·1 |
| Rest 1st hour after walk | 22 | 35·9 |
| „ 2nd „ | 36 | 100·3 |

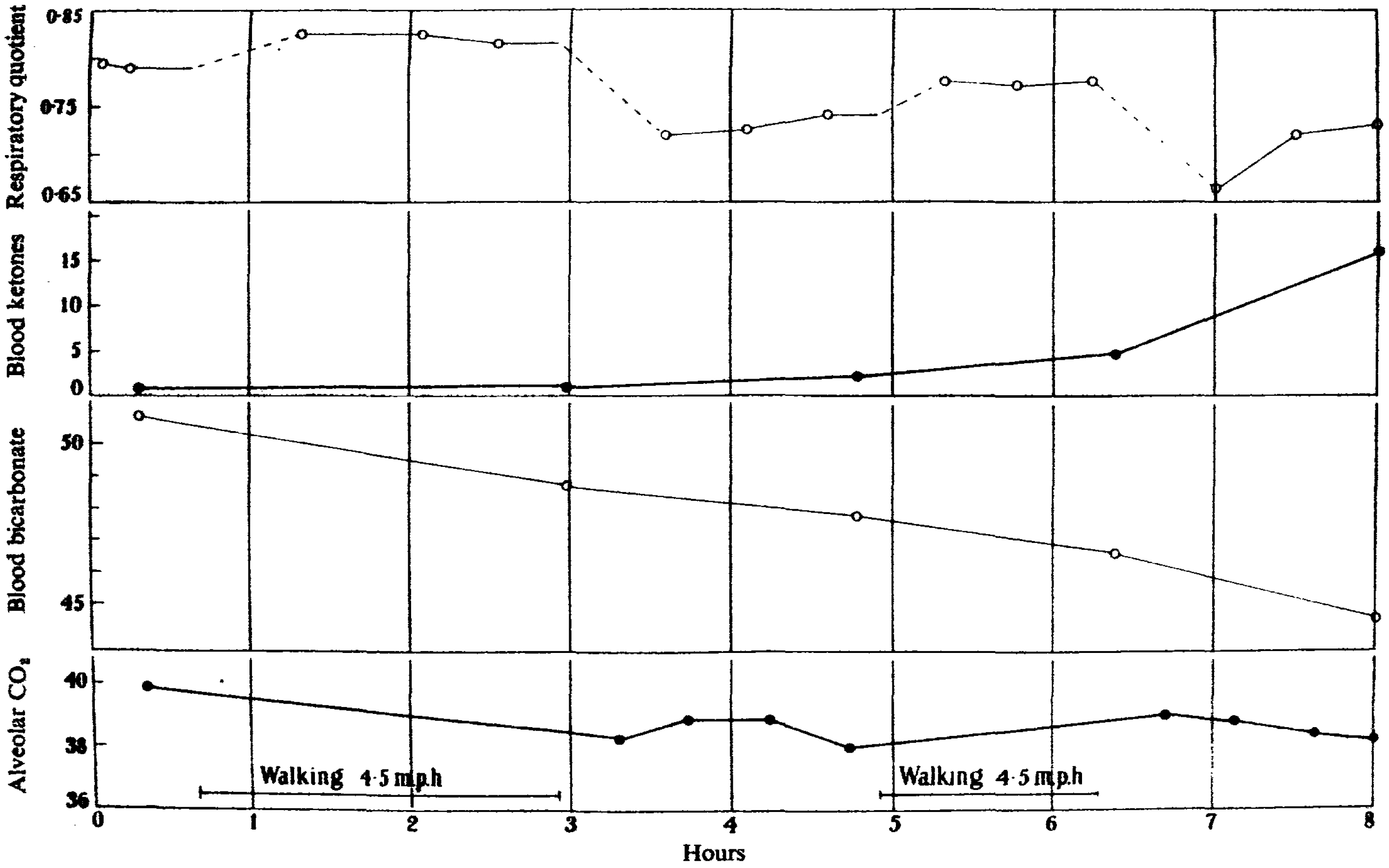


FIG. 8—Douglas, post-absorptive. Effect of walking 10 miles at 4·5 miles per hour followed by a further walk of 6 miles at the same pace after an interval of rest of 2 hours. Blood ketones expressed as mg β-hydroxybutyric acid %. Blood bicarbonate = $CO_2$ combining power of blood in volumes %. Alveolar $CO_2$ pressure in mm Hg.

ketone bodies during rest after the first walk and particularly after the second walk. The diminished excretion during the second walk must be dependent on the greatly reduced secretion of urine at this time. The diuresis which occurs before the walks, and in the interval between them, is due to the ingestion of fluid.

The average respiratory exchange of Courtice in two similar experiments is shown in Table XXVI. The course of events is very much the same as that shown in fig. 7. Ketonuria was absent during the preliminary rest and the first walk, but present throughout the remainder of the experiment, although the Rothera reaction seemed to be least marked in the urine secreted during the second walk. In one of these experiments no ketone bodies could be detected in the blood at the end of the first walk, but 1·3 mg per 100 cc were recovered in a sample of blood taken after resting for 1½ hours before beginning the second walk. The concentration of ketone bodies in a blood sample taken just after the end of the second walk was still only 1·1 mg per 100 cc, whereas after resting for a further 1½ hours the concentration had risen to 11·5 mg

TABLE XXVI—COURTICE, POST-ABSORPTIVE. TEN-MILE WALK AT 4·5 MILES PER HOUR FOLLOWED BY A FURTHER WALK OF 6 MILES AFTER AN INTERVAL OF REST OF 2 HOURS

| | Respiratory exchange cc/min | | R.Q. |
|---|---|---|---|
| | $CO_2$ | $O_2$ | |
| Rest before walk | 176 | 219 | 0·80 |
| „ | 183 | 223 | 0·82 |
| End of 2 miles' walk | 1285 | 1570 | 0·82 |
| „ 4 „ | 1261 | 1556 | 0·81 |
| „ 6 „ | 1260 | 1567 | 0·81 |
| „ 8 „ | 1242 | 1558 | 0·80 |
| „ 10 „ | 1262 | 1616 | 0·78 |
| Rest ½ hour after walk | 172 | 230 | 0·75 |
| „ 1 „ | 172 | 229 | 0·75 |
| „ 1½ „ | 182 | 234 | 0·78 |
| End of 2 miles' walk | 1253 | 1611 | 0·78 |
| „ 4 „ | 1241 | 1602 | 0·78 |
| „ 6 „ | 1245 | 1612 | 0·78 |
| Rest ½ hour after walk | 178 | 243 | 0·73 |
| „ 1 „ | 190 | 252 | 0·75 |
| „ 1½ „ | 188 | 251 | 0·75 |

per 100 cc. The concentration of ketone bodies in the blood had therefore not been increased during the second walk above the figure reached during the intermediate rest period.

In these experiments the subject rested for 2 hours after a 10-mile walk before beginning the second walk. Ketone bodies accumulated in the blood during this rest period, and their concentration might then remain unaltered during the second walk or increase slightly, but they did not disappear. We therefore tried what would happen if a short interval of rest were interposed at earlier intervals during the course of a walk. We knew that we never developed ketonuria during an uninterrupted 10-mile walk, and we found that this was also true if we increased the length of the walk to 12 miles. On the other hand, if we rested for half an hour at the end of every fourth mile ketonuria appeared much earlier, and this was true of both subjects. Thus urine secreted during the first 4 miles, during a rest period of half an hour and during the next 4 miles' walk gave a negative Rothera reaction. During the ensuing half-hour's rest the Rothera reaction became positive, and continued positive in urine secreted during the third 4 miles' walk, becoming intense during a final resting period. It looked therefore as though ketonuria, when once it had developed during rest, could not be abolished by further exercise.

## BLOOD SUGAR AND SUGAR TOLERANCE

Blood sugar determinations were made in a number of experiments during the pre-exercise and post-exercise resting periods. Some examples of these are given in Table XXVII. In experiments A and B on Courtice and in C on Douglas the subject was in the post-absorptive state and had had his normal diet on the day before. Experiment D on Douglas is interesting as it is the experiment of which data are given in Table XXII. On this day he had had his normal breakfast but the post-exercise respiratory quotient fell to, and remained at, an exceptionally low level. In none of these experiments does the blood sugar show any significant change after the exercise, but always remains within the normal limits. Evidently the amount of exercise which we took was insufficient to affect the blood sugar level, although this may be affected if the exercise is more severe and prolonged. Thus Edwards, Margaria, and Dill (1934) found that if work was done at a rate corresponding to an oxygen consumption of 2 to 2·5 litres per minute, with a pause of 5 minutes at the end of every half-hour, the blood sugar did not fall until the exercise had been continued for 3 to 4 hours when the subject was becoming exhausted. Dill, Edwards, and Talbott (1933) have shown, too, that in the dog the

TABLE XXVII—EFFECT OF A 10-MILE WALK AT 4·5 MILES PER HOUR ON THE RESTING BLOOD SUGAR. EXPERIMENTS A, B, AND C, SUBJECTS POST-ABSORPTIVE; EXPERIMENT D, NORMAL BREAKFAST BEFORE EXPERIMENT

| *Courtice* | Blood sugar mg/100 cc | |
|---|---|---|
| | Exp. A | Exp. B |
| Initial rest ........ | 94 | 89 |
| Rest after work for— | | |
| 1 min. ........ | 86 | 83 |
| 5 " ........ | 89 | 94 |
| 15 " ........ | 95 | 93 |
| 25 " ........ | 96 | 87 |
| 55 " ........ | 97 | — |
| 85 " ........ | — | 87 |
| 100 " ........ | 97 | — |
| 118 " ........ | 94 | 101 |
| 140 " ........ | 101 | — |
| 172 " ........ | — | 87 |
| 190 " ........ | 93 | — |
| 217 " ........ | — | 90 |

| *Douglas* | Blood sugar mg/100 cc | |
|---|---|---|
| | Exp. C | Exp. D |
| Initial rest ........ | 99 | 101 |
| Rest after work for— | | |
| 1 min. ........ | 92 | 102 |
| 43 " ........ | 91 | 93 |
| 70 " ........ | 94 | 94 |
| 101 " ........ | 98 | — |
| 134 " ........ | — | 93 |
| 164 " .......... | — | 95 |
| 194 " ........ | — | 95 |

blood sugar does not fall until severe muscular work has been done for a long time.

For Courtice we have also tested the sugar tolerance both before and after exercise. Fifty gm of sucrose were taken in solution in water with a little lemon juice in the post-absorptive state after resting for half an hour, and the blood sugar was determined every quarter of an hour whilst resting for 2 hours. On another day the same amount of sucrose was taken after resting for 2 hours following a walk of 10 miles, and the blood sugar was determined during the ensuing 2½-hour rest. Fig. 9 gives the average results of four experiments before and four experiments after exercise. It will be seen that after exercise the blood sugar concentration rises rather more slowly and falls to a normal level more gradually. In other words there is evidence of a reduced sugar tolerance.

In fig. 10 the influence is shown of the ingestion of 50 gm of either sucrose, glucose, or fructose on the respiratory quotient during rest when these sugars are taken by the post-absorptive subject in the first instance after a preliminary rest period of half an hour, and in the second 1 to 2 hours after he had finished a 10-mile walk.

The respiratory quotient before taking the sugar is considerably lower in the experiments made after exercise than in those when no muscular work had been done. Even if we make allowance for this, the absolute change of respiratory quotient after taking either sucrose or glucose is less in the experiments made after work, and the rate at which the quotient rises is slower. The same effect is less prominently shown after taking fructose. With both sucrose and glucose the highest value of the respiratory quotient is maintained longer in experiments made after work than in those in which no work was done. The lag in the rise of the respiratory quotient which is shown when the sugar is ingested during rest after exercise cannot be due to slow absorption of the sugar, since fig. 9 shows that at the end of half an hour the blood sugar is actually higher when the sugar is ingested after exercise than when no exercise is taken.

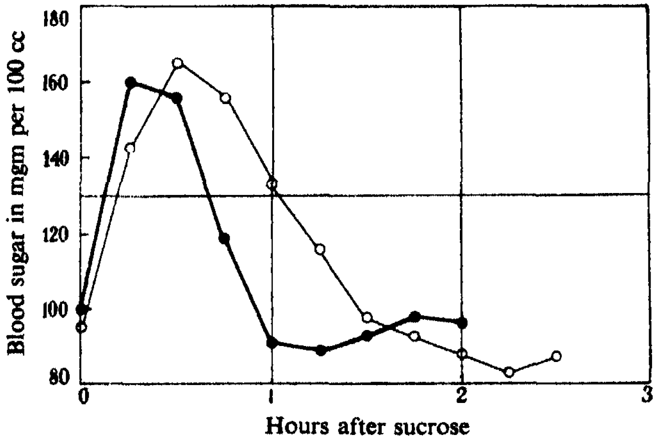


FIG. 9—Courtice. Effect of ingesting 50 gm of sucrose on the blood sugar before and after walking 10 miles at 4·5 miles per hour. ● Before exercise; ○ after exercise.

## DISCUSSION OF THE RESULTS

During the work we were evidently in what is termed the "steady state". The walk was done at a reasonably uniform rate throughout. Our own analyses and alveolar $CO_2$ estimations, as well as Owles's earlier observations on Douglas, make us confident that changes in the respiratory quotient during the walk in our experiments cannot be attributed to material changes in the lactic acid content of the blood. The period during which the body temperature was actually rising as a result of the exercise was limited to about the first half-hour of the walk, and thereafter the body temperature remained steady. We began the first determination of the respiratory exchange during the work after we had been walking for about 22 minutes. The body temperature may still have been rising at this time, but the respiratory quotient during the last 1¾ hours of the walk must have been unaffected by body temperature since this remained steady during this period. We have shown, too, that ketonuria never developed during the walk. Excluding therefore lactic acid, body temperature, and ketosis as factors affecting the respiratory

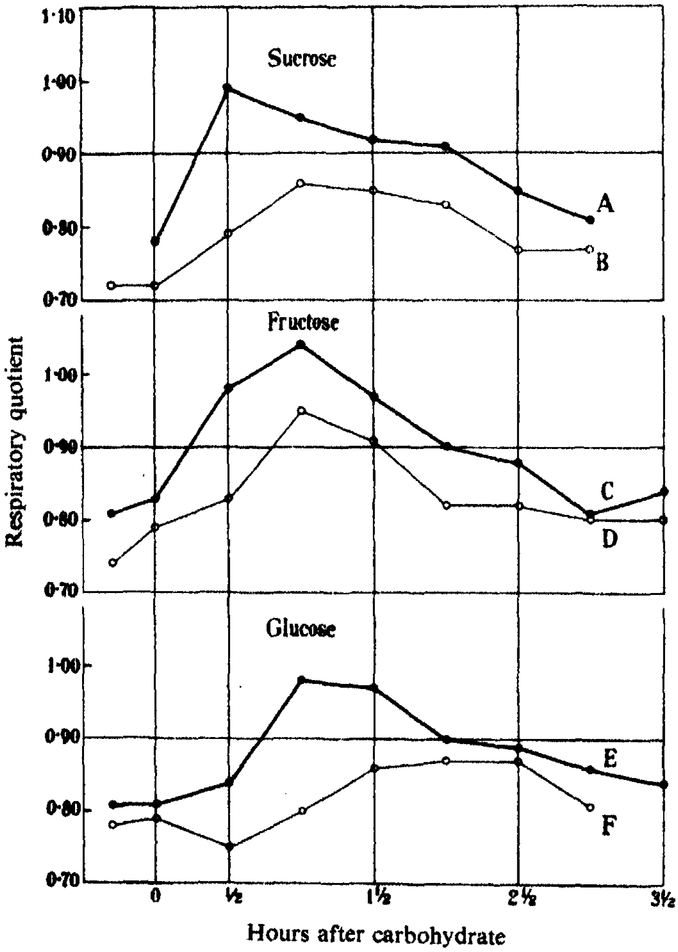


FIG. 10—Courtice. Effect on the respiratory quotient of 50 gm of sucrose, glucose, or fructose before and after walking 10 miles at 4·5 miles per hour. ● Before exercise; ○ after exercise.

quotient during the work we are left with the conclusion that this quotient must depend on the ratio of the substances oxidized to supply energy, for it hardly seems likely that at a time when the rate of oxidation is greatly increased the quotient can be affected by the conversion of carbohydrate into stored fat, or vice versa, so long at least as the quotient is in the neighbourhood of 0·8. It has already been pointed out that if we accept the respiratory quotient as a true oxidation quotient it means that during the walk about 100 gm of carbohydrate must have been used in those experiments made on the post-absorptive subject who had been taking his ordinary diet on the previous day.

So far as the actual respiratory exchange during the work is concerned our figures agree with what has been found by other observers in work of similar severity. Although much of the earlier work on the respiratory exchange during muscular exercise, *e.g.*, that published by the schools of Zuntz and Durig, seemed to show that moderate work had little influence on the respiratory quotient, more recent investigations on subjects who were either post-absorptive or taking their ordinary diet have established the fact that the respiratory quotient tends to rise at first during work of this type, and perhaps to fall slowly if the work is long continued. This is true, for instance, of the observations made by Amar (1910), Douglas, Haldane, Henderson, and Schneider (1913), Benedict and Cathcart (1913), Benedict and Murschhauser (1915), Campbell, Douglas, and Hobson (1920), and Smith (1922) and the conclusion has therefore been drawn that there is a preponderating use of carbohydrate as a source of energy during the exercise. Krogh and Lindhard (1920), in their investigation of the relative value of fat and carbohydrate as sources of muscular energy, found that if the respiratory quotient was very high it tended to fall when work was begun, and if it was low it tended to rise, there being little change when the resting quotient was between 0·8 and 0·9.

Our own observations agree with those of Krogh and Lindhard so far as we find that with low initial respiratory quotients the change of quotient during the work is greater than when the initial quotient is higher, but in our experiments even when the initial quotient is somewhat above 0·8 there may still be a distinct rise, at least in the earlier periods of the work. This is shown in fig. 11, which gives the results on both subjects in the post-absorptive state, the higher initial quotients being due to an increase in the carbohydrate taken on the previous day. The upper series of points show the change in respiratory quotient when the initial resting figure is compared with the first determination made during the work; in the lower series of points the initial resting figure is compared with the average respiratory quotient during the whole of the work.

In Krogh and Lindhard's experiments the respiratory quotient during the work varied somewhat irregularly, but on the whole there is a slight tendency towards a fall, and they conclude that the organism maintains a remarkably constant proportion between the amounts of carbohydrate and fat katabolized, this proportion being a function of the available supplies of the two sources of energy. The change in quotient shows that the balance between carbohydrate and fat is very slowly altered as the work progresses in favour of the latter.

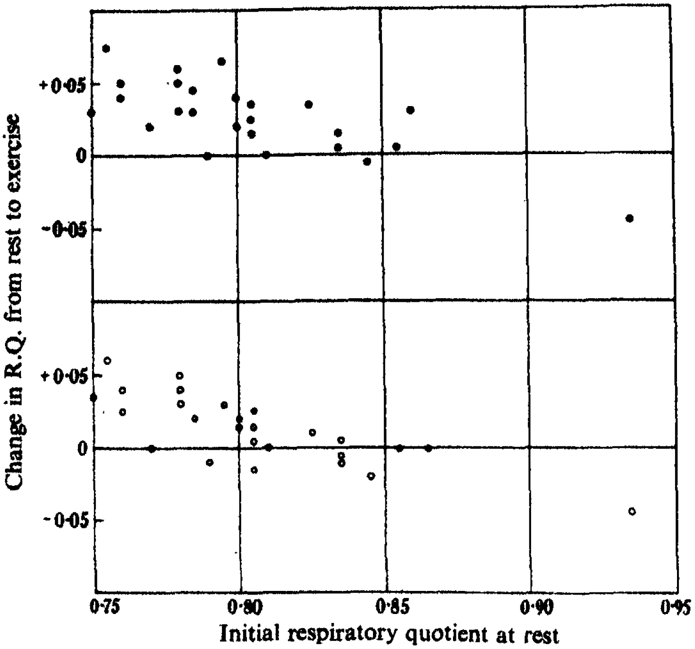


Fig. 11—Alteration in the initial respiratory quotient caused by walking at 4·5 miles per hour. ● Change produced during early period of walk; ○ change produced taking average R.Q., during whole walk of 10 miles.

The work which we did in our experiments was more severe, and lasted longer, than that in Krogh and Lindhard's experiments, and our actual determinations of the respiratory exchange were spread over a longer period of work. It is not surprising therefore that we got more evidence of a fall of respiratory quotient during the course of the work. A similar fall is apparent in the more recent observations of Christensen, Krogh, and Lindhard (1934) on the effect of severe and prolonged athletic exercise. We agree that the respiratory quotient must depend largely on the availability of carbohydrate at the time, and that as the work continues this availability becomes reduced owing very likely to the gradual depletion of glycogen stores in the liver. In the later stages of work we may expect therefore to have a much smaller proportion of carbohydrate to fat metabolized than in the earlier stages, and we have given on p. 394 figures to show this.

It does not of course follow that all individuals will react in precisely the same way, for " availability " is a general term which must include such factors as the influence of the diet previously taken, as well as individual differences in the capacity for storing and possibly for mobilizing carbohydrate. Another factor which may come into play is the influence of differences in the rate at which the work is done. Even when we restrict ourselves to work which may be regarded as light or moderate in character there is evidence that if the respiratory quotient increases during the work the rise is more evident at the faster rates of work. If the work is definitely hard for the subject there may of course be a considerable rise of the respiratory quotient, but in such a case it is very likely to be due in part to the accumulation of lactic acid. In the experiment detailed in Table XVII in which the pace of the walk was reduced to 3½ miles per hour there is no rise of the respiratory quotient even in the earlier stages of the walk, and at most but a slight fall as the work continues. The total amount of work done (as judged from the oxygen consumption) is, however, just the same as in the experiments done at the pace of 4½ miles per hour. Yet the calculated consumption of carbohydrate during the walk in this experiment is only 71 gm as compared with the figure of about 100 gm found in experiments at the faster pace.

A reduction of 100 gm in the available carbohydrate in the body may at first sight appear too small to matter seriously, but the significance of this reduction must depend on the amount of carbohydrate originally available, and it is impossible to determine what this amount is. The old statement that the liver of a well-nourished man may contain 150 gm of glycogen, and that there is about an equal amount in the muscles, cannot be accepted as it stands in view of more recent determinations of the glycogen stores in animals under different conditions. Junkersdorf (1925) fed dogs on a diet similar to Voit's standard diet for man and found an average of 6·1% of glycogen in the liver and 0·55% in the muscles. Schöndorff (1903), feeding dogs on a carbohydrate rich diet, found a glycogen content in the liver of 7·3–18·7%, and in the muscles of 0·7–3·7%, while Junkersdorf (1921, *b*) showed that the glycogen content of the dog's liver may reach 16·4% with excessive feeding with protein and carbohydrate. Assuming that these figures can be applied to man, and that a man weighing 70 kilos has a liver weighing 1·5 kg and muscles weighing 21 kg (30% of his body weight), the glycogen content of the liver might vary from 92 gm to 280 gm, and of the muscles from 115 gm to 777 gm. Whatever a reasonable estimate of the glycogen stores of a normal man may be, it seems evident that we may encounter extreme variations according to the amount of carbohydrate taken in the diet.

It has long been known that starvation coupled with muscular work leads to a very rapid reduction of the glycogen in the liver of experimental animals, as well as to a more gradual reduction of muscle glycogen. Junkersdorf (1921, *a*) found that after starving dogs for 11 days the glycogen content of the liver was 0·59% and of the muscles 0·21%. Comparing these figures with those given above for dogs on the Voit standard diet the glycogen content of the muscles has been considerably diminished, but that of the liver has been reduced in much greater proportion. The greater ease with which the glycogen content of the liver can be depleted in comparison with that of the muscles is also shown after the administration of phlorhizin (Junkersdorf, 1922, 1923) or pancreatectomy (Fisher and Lackey, 1925; Chaikoff, 1927). Bollman, Mann, and Magath (1925) have shown that after extirpation of the liver the normal level of blood sugar cannot be maintained at the expense of the glycogen in the muscles, while Best, Hoet, and Marks (1926) have shown that an insulin hypoglycaemia does not appreciably lower the glycogen content of the skeletal muscles of the spinal animal unless insulin convulsions are caused.

All these facts seem to show that although glycogen may be readily given up by the liver to supply the needs of the body the muscles tend to maintain their glycogen content, presumably because its presence is intimately associated with their normal activity. If therefore in our experiments the brunt of the supply of carbohydrate demanded by the active muscles has to be borne by the liver, assuming that the form of exercise which we have adopted was not sufficiently strenuous or prolonged to cause a significant diminution of muscle glycogen, the loss of something like 100 gm of liver glycogen may well have been material, especially if the liver at the start had a glycogen content well below the maximum. We can therefore understand that as the work continues an increasing call must be made upon fat to supply the energy required.

When we took our normal diet on the day preceding the experiment our initial respiratory quotients were about 0·81 and 0·79, but when we had deliberately taken a diet rich in carbohydrate on the preceding day the initial quotients were 0·93 and 0·86. In one of the latter experiments on Courtice, Table XIX, the calculated consumption of carbohydrate during the walk was 153 gm and in another on Douglas 141 gm, *i.e.*, greatly in excess of the average figure of about 100 gm obtained when the subjects had been living previously on their normal diet, whilst the resting respiratory quotients after exercise did not fall below the figures found during pre-exercise rest when the diet taken previously had been normal in character. This suggests to us that when taking his normal diet neither subject had made anything like full use of the storage capacity of his liver for glycogen.

The calculated consumption of carbohydrate may reach far higher figures for good athletes when the exercise is much more severe and prolonged than in our experiments. Thus Benedict and Cathcart's post-absorptive subject M. A. M. consumed 368 gm of glycogen during work on the bicycle ergometer lasting for 4 hours 22 minutes, which he regarded as equivalent to cycling 100 miles on the road. It may be noted that the average respiratory quotient during pre-exercise rest in the post-absorptive state was about 0·85 in this subject, so he very likely had a larger initial supply of available carbohydrate than we had on our normal diets. Christensen, Krogh, and Lindhard (1934) record that a subject on a diet extremely rich in carbohydrate can consume 400 gm of carbohydrate during work on the bicycle ergometer for 4 hours at a rate of 1080 kg m per minute, which would involve an oxygen consumption of about 2800 cc per minute, whilst his respiratory quotient fell during the course of the work from 0·91 to 0·82. In this case the subject apparently worked to exhaustion, and there is nothing, of course, to show the degree to which the glycogen stores of the muscles were implicated as well as those of the liver. The important part played by carbohydrate in maintaining the capacity of a dog to do very severe muscular work for many hours has been strikingly shown by Dill, Edwards, and Talbott (1933).

Turning now to the period of rest after the work we have to consider the significance of the persistent low respiratory quotient. The excess lactic acid found in the blood at the end of the work is so insignificant in amount that its elimination can hardly play any part as a cause of the low quotient in the early stages of rest. We are inclined to attribute the abnormally low quotient seen in Douglas at the end of the first half-hour's rest to the influence of falling body temperature. The main cause of the lowering of the respiratory quotient must depend on a genuine difference of the metabolism from that which characterized the pre-exercise period. A natural suggestion to make is that, if we are right in supposing that the work did result under the conditions of our experiments in a substantial depletion of the available carbohydrate, when the metabolism falls to its resting level again a far greater proportion of fat to carbohydrate will be used than in the pre-exercise period. This explanation is strongly supported by the ketosis which develops very quickly after the work stops and slowly increases as time goes on. Ketosis is generally believed to occur when the ratio of carbohydrate to fat metabolized sinks below a certain level. It seems to be agreed that when the metabolism of fat to carbohydrate (*i.e.*, ketogenic to anti-ketogenic substances) is in the molecular ratio of 1 : 1 there is little or no ketosis, though the ketosis becomes marked if this ratio rises to 2 : 1. In Table VII it was shown that Courtice has an average respiratory exchange of 181 cc of $CO_2$ and 237 cc of oxygen, with a respiratory quotient of 0·76, during the post-exercise rest period. Allowing for a protein metabolism of 3·3 gm per hour it can be calculated by the methods of either Woodyatt (1921) or Shaffer (1921, 1923) that there would be a ratio of ketogenic to anti-ketogenic substances of about 1·1 : 1. A post-exercise respiratory quotient of 0·76 was the highest with which we invariably had a ketosis, though, as has been pointed out earlier, we sometimes got ketonuria with a quotient of 0·77 and occasionally we could detect a faint positive Rothera reaction with a quotient as high as 0·78.

Another question which obviously needs consideration is whether the persistent low respiratory quotient during post-exercise rest is influenced by the conversion of either protein or fat to carbohydrate, and the storage of the latter in the liver and possibly the muscles. If the respiratory quotient were to remain at so low a level that it could not be explained by the simultaneous oxidation of protein and fat this might be regarded as evidence in favour of this conversion, provided that the incomplete combustion of fat with the production of ketone bodies were insufficient to account for the facts. If, for example, one molecule of β-hydroxybutyric acid is formed from one molecule of oleic acid while the remainder of the latter molecule is oxidized to $CO_2$ and water the respiratory quotient of the reaction will be 0·667, whereas if the oleic acid molecule were completely oxidized to $CO_2$ and water the quotient would be 0·707.

Unfortunately, as is so common in experiments of this type, our figures do not afford indubitable evidence of this transformation of fat into carbohydrate. Thus in Table IX it is shown that Douglas's average post-exercise respiratory quotient is 0·73, and that if we make what seems a fair maximum allowance for the protein metabolized the quotient sinks to 0·71, and we must admit that this could be explained simply by the complete oxidation of fat even if we do not regard such an explanation as probable. In one experiment on Douglas given in Table XXII we record a persistent post-exercise respiratory quotient of 0·71, which, if allowance is made as before for the protein metabolism, might be reduced to 0·68, but this is the only instance in which we got a persistent quotient below 0·7.

In those experiments in which we measured the amount of ketone bodies excreted in the urine the average output did not exceed 100 mg per hour, *cf.* for instance Table XVI. The formation of 1 gm of β-hydroxybutyric acid requires an uptake of 108 cc of oxygen without corresponding liberation of $CO_2$, and consequently the formation of 100 mg of this acid per hour would require an oxygen uptake of 10·8 cc or 0·18 cc per minute. Admittedly these estimations of the rate of excretion of ketone bodies were made in experiments in which the respiratory quotient was a good deal higher than in the exceptional instance given above, and we ought to take into account the fact that the rate of excretion of ketone bodies in the urine does not necessarily show the rate of their accumulation in the body, but so far as we can judge we need not consider the formation of ketone bodies seriously as a cause of the low respiratory quotients in our experiments.

As it is difficult to get conclusive evidence by this line of argument we have tried to arrive at a figure which would represent the maximal rate of conversion of fat into carbohydrate which is compatible with the observed respiratory exchange and quotient during post-exercise rest in our experiments. In order to do this we have assumed that the energy liberated by the subject during rest is the same, no matter whether work has or has not preceded the rest, and is derived from the oxidation of precisely the same mixture of protein, carbohydrate, and fat, and that if the observed respiratory quotient falls during the post-exercise period this is due merely to a concurrent conversion of fat into carbohydrate with storage of the latter. If we take the figures for Courtice given in Table VII we see that his $CO_2$ output is the same during rest after exercise as it was before, but that his oxygen consumption is 14 cc higher, so that his respiratory quotient has fallen from 0·81 to 0·76. Let us take it for the moment that this constant output of $CO_2$ represents the rate at which energy is being liberated. Assuming for argument that the whole of the carbon in the fat molecule can be converted into carbohydrate by the uptake of oxygen (though we admit, of course, the weakness of any such empirical equation) we should get for triolein

$$2C_{57}H_{104}O_6 + 46O_2 = 19C_6H_{10}O_5 + 9H_2O,$$

and an uptake of 1 litre of oxygen would convert 1·7 gm of fat into 3 gm of carbohydrate. By this method of reckoning the extra 14 cc of oxygen consumed by Courtice per minute during the post-exercise period above the quantity necessary to give the same respiratory quotient as during the pre-exercise rest, viz., 0·81, would represent the conversion of 1·5 gm of fat into 2·6 gm of glycogen per hour.

It is less easy to make a similar calculation for Douglas since, as will be seen from the average figures given in Table VII, not only is the post-exercise consumption of oxygen higher than during the pre-exercise period, but, in addition, the post-exercise output of $CO_2$ is less than the pre-exercise. The simplest thing to do is to assume as above that the $CO_2$ output indicates the rate of energy liberation, and that the lower value during rest after work is due merely to the fact that the degree of rest was more complete after the exercise than before it. If during the post-exercise period the respiratory quotient had been the same as in the pre-exercise period, viz., 0·79, the $CO_2$ output of 176 cc would have corresponded with an oxygen consumption of 221 cc and the oxygen consumption actually observed would have been 19 cc greater than this figure. This would correspond to the conversion of 2·0 gm of fat into 3·5 gm of glycogen per hour. Even if we take the exceptional instance shown in Table XXII it would only require the conversion of 2·8 gm of fat into 4·9 gm of carbohydrate per hour to change a quotient of 0·79 into the observed figure of 0·71.

Of course a calculation of this type must not be pressed too far. In making it we have deliberately discounted any alteration of the ratio of fat and carbohydrate burned, nor have we considered the possibility of the conversion of protein into carbohydrate, although the more there is of this the less will be the conversion of fat. But the point that we wish to make is really this, that so far as we can see any conversion of fat or protein into carbohydrate with storage of the latter must have been at a very slow rate. We do not mean to imply that such a conversion cannot take place, but that if it does the rate is insufficient to lead to a material replenishment of the stores of available carbohydrate in the liver or elsewhere. We feel that this is supported by the fact that the low respiratory quotient was maintained steady in the last 2 hours of post-exercise rest while the ketosis was increasing, and that even when the post-exercise rest period was prolonged to 9½ hours there was no indication that the quotient was rising towards its normal value.

Poulton (1933) has recently drawn attention to the fact that in many of the published observations on the basal metabolism there is a discrepancy between the heat output directly determined in the calorimeter and that estimated from the respiratory exchange at low and high respiratory quotients, agreement being only reached with a respiratory quotient of about 0·785. This might be regarded as evidence in favour of the conversion of carbohydrate to fat at high quotients and of fat to carbohydrate at low. If we calculate the calorie output by means of the Zuntz-Schumburg table in our experiments, Courtice has on the average a somewhat higher calorie output during rest after exercise than in the initial rest period, viz., 1·13 calories per minute as opposed to 1·07. Douglas on the other hand gives a post-exercise figure of 1·13 calories and a pre-exercise of 1·10. These differences are hardly large enough to be significant. If we plot out, as we have in fig. 12, the results of individual experiments it will be seen that for Courtice there does seem to be a definite tendency for the calculated calorie output to be higher at the low quotients which prevail after exercise, these low quotients being dependent on an increase in oxygen consumption without any definite variation in the $CO_2$ output. The scatter of the individual points for Douglas is too great to justify any definite conclusion. Again, therefore, evidence in favour of a transformation of fat into carbohydrate must be regarded as inconclusive.

Occasionally we felt chilled when sitting still for a long time after the exercise was over, and this may have prevented the metabolism from falling as low as at other times, but apart from this we can see no reason why the metabolism after the work should not be as low as during the pre-exercise period, if not lower: at all events we were apt almost to fall asleep during the long rest after the exercise.

In view of the occurrence of ketosis and of the behaviour of the respiratory quotient during a second period of exercise in our double walk experiments, which will be discussed below, we are inclined to attribute the cause of the low post-exercise respiratory quotients far more to an increase in the ratio of fat to carbohydrate oxidized than to the conversion of fat into glycogen.

In our experiments on the effects of the ingestion of carbohydrate before doing the work we have clearly met the same phenomenon as that described by Christensen, Krogh, and Lindhard (1934) in their investigations on severe and prolonged athletic exercise, viz., that a diet rich in carbohydrate taken on the day preceding the exercise may in certain respects have a greater influence than the ingestion of a quantity of easily assimilable carbohydrate either during, or shortly before beginning, the work. They conclude that " when long continued, severe work is to be performed the preceding diet must provide ample energy, mainly in the form of carbohydrate, to fill up the stores of glycogen. When the absolute maximum of work is to be attained . . . about 2 days' rest is required to secure a complete filling up of the glycogen stores, and it appears that this cannot be done by taking large amounts of carbohydrate just prior to the work ".

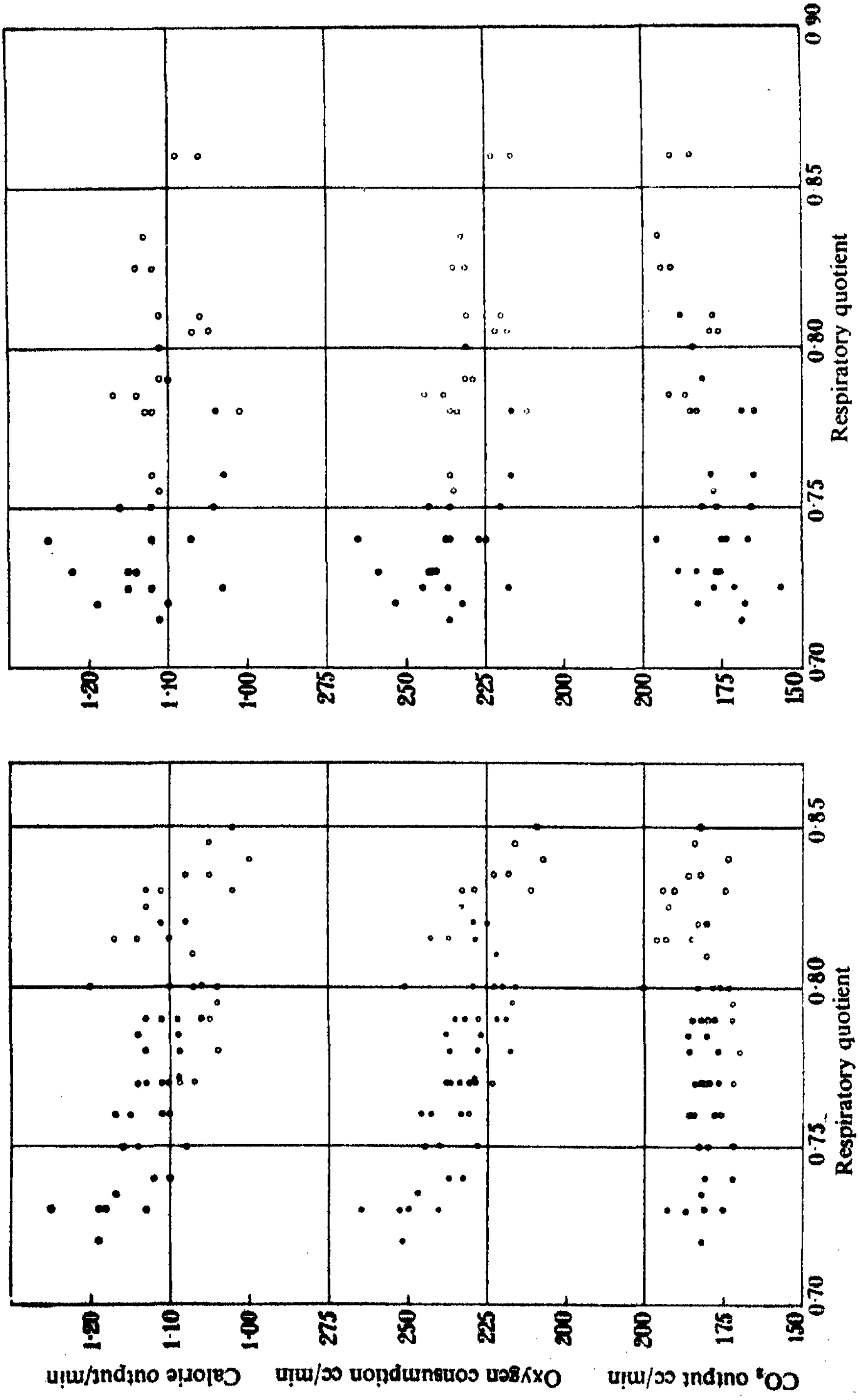


FIG. 12—The relation between the R.Q. and the CO$_2$ output, oxygen consumption, and calculated calorie output with the subject resting. O Before exercise; ● after exercise. The left-hand side of the figure refers to Courtice, the right-hand side to Douglas.

In our own experiments, when we took a predominantly carbohydrate diet on the day preceding the experiment, the experiment itself being made on the post-absorptive subject, the general behaviour of the respiratory exchange was very much the same as when the subject had been previously taking his normal diet, save that the respiratory quotient ran throughout at a higher level and ketonuria failed to appear during the period of rest succeeding the exercise. On the other hand when we took carbohydrate, mainly in the form of glucose or fructose, shortly before beginning the experiment post-exercise ketonuria developed in 9 out of 12 experiments. We only took 60–70 gm of carbohydrate in these experiments, but, assuming that in the normal post-absorptive experiments about 100 gm of glycogen was used up during the exercise, we felt that the ingestion of this moderate amount of carbohydrate might possibly so far compensate for this loss of glycogen as to make a substantial difference during the post-exercise rest. What actually happened was that the respiratory quotient, which was very high at the beginning of the work, fell rapidly and steadily so that as the work neared its end it was not very different from the figure found in the normal post-absorptive experiments. If we make a straightforward calculation of the carbohydrate used up during the work it would appear that the only result of the ingestion of the carbohydrate just before beginning the experiment is to exaggerate the use of carbohydrate during the work to such a degree that when the work stops we have much the same condition as in the normal post-absorptive experiments, and as a result we still find a post-exercise ketonuria. Such a calculation neglects the possibility of the transformation of carbohydrate into stored fat, and though we may feel that such a transformation is improbable when the respiratory quotient is in the neighbourhood of 0·8 we cannot disregard this as a possibility when the quotient approaches and even exceeds 0·9, for it may be that when there is a sufficient surplus of carbohydrate available some of this may be converted into fat, even though the metabolism of the muscles is greatly increased at the time. Attention has also been drawn to the accentuation of carbohydrate metabolism during muscular work when glucose or fructose has been ingested beforehand by Carpenter and Fox (1931), although the exercise in their experiments was less severe and of shorter duration than in ours.

When the carbohydrate was ingested shortly before the beginning of the experiment the work must, we feel, still have resulted in a very material depletion of the body's stores of " readily available " carbohydrate if we are to explain the low quotient and the ketosis which persist after the exercise is over—a depletion which is not nearly so apparent when we took a carbohydrate diet on the previous day, presumably because this gave a better opportunity for increasing the stores of available carbohydrate, very likely in the form of glycogen in the liver.

Depleted though the stores of available carbohydrate may have been in our normal post-absorptive experiments, and even in those in which we ingested carbohydrate just before beginning the experiments, they were by no means exhausted. This, we think, is clearly shown by the results of the double walk experiments. We know that a single 10-mile walk always resulted in a low respiratory quotient which persisted for hours and was accompanied by an increasing ketosis. If, however, after 2 hours' rest we walked for a further 6 miles the quotient at once rose considerably, and remained steady at this higher level until the end of the walk when it dropped abruptly again to a low level. It is noteworthy, too, that though a ketosis developed in the interval between the two walks this ketosis either showed no further change, or but a slight increase, during the second walk, although in the final period of rest it became very marked.

We have not made any determinations of the lactic acid in the blood at the end of the second walk, but since there was hardly a perceptible increase at the end of a single 10-mile walk it seems unlikely that a further walk at the same pace after an interval of 2 hours' rest would have given a different result. Using the arguments which have already been developed about the significance of the respiratory quotient during a single 10-mile walk we feel that the steady respiratory quotient during the second walk gives an index of the proportions of substances being oxidized at the time, and that during this period a larger proportion of carbohydrate to fat is being used than in either the intermediate or the final rest periods. Taking the data for Douglas which are shown in fig. 7 the oxygen consumption during the second walk was about 1730 cc per minute and the respiratory quotient 0·78. Neglecting the protein metabolism during the walk these figures would correspond to an oxidation of 0·486 gm of carbohydrate and 0·667 gm of fat per minute, and the total amount of carbohydrate used up during the 80 minutes' walk would be 39 gm and of fat 53 gm. The amount of carbohydrate used is substantial, but the proportion of energy derived from carbohydrate to that derived from fat is now only about 1 : 3.

If this is so, a pertinent question at once suggests itself. Why is it that the predominantly fatty metabolism which characterizes the intermediate period of rest should so abruptly change with the onset of the second period of work into a type in which carbohydrate plays a more prominent part, and that there should be an equally abrupt reverse change in the final period of rest? It is not easy to answer this question, but there are certain facts which seem suggestive.

In the experiments made on Courtice there is evidence of a reduced sugar tolerance during the post-exercise rest period. The sugar tolerance curve has been frequently used as an index of the power of the tissues to use glucose, either by storing it as glycogen or fat, or by burning it, and the curves shown in the experiments on Courtice closely resemble the tolerance curves obtained after starvation or high fat diets by previous workers. Staub (1922) found that the sugar tolerance curve was lower on a fat diet than on a carbohydrate diet, and to account for this postulated a reduced output of insulin from the pancreas on the former diet. Sweeney (1927) studied the effects of starvation and of fat, protein, and carbohydrate diets, and concluded that the higher sugar tolerance on a carbohydrate diet was due to a greater sensitization of the insulin secreting mechanism, with a resultant increase in the secretion of insulin, an explanation with which Macleod (1930) agreed. On the other hand Dann and Chambers (1930) have shown that in dogs which have fasted from 15 to 30 days the administration of glucose causes little rise of the respiratory quotient, and that when insulin is injected at the same time as the administration of glucose very little more glucose is oxidized, showing apparently that the reduced sugar tolerance in these conditions is not merely due to lack of insulin. More recently Himsworth (1934) has suggested that fat diets cause a decreased susceptibility of an animal to insulin, and carbohydrate diets the reverse, possibly because insulin is secreted in an inactive state and has to be activated in the body by some unknown substance which is increased by a carbohydrate diet and decreased by a fat diet. We have clearly much to learn before we shall understand the mode of action of insulin.

It is quite likely that an increased secretion of insulin plays a part in causing the greater use of carbohydrate during the performance of the work when that work is done soon after ingesting carbohydrate, but though a mere surplus of carbohydrate gaining access to the body may be effective in causing increased insulin production, or in bringing about an enhanced effect of that substance, there may well be some other factor at work in addition. If, for instance, the activity of the islets of Langerhans—and, no doubt we ought to add, of the other endocrine organs associated with the general metabolism of carbohydrate in the body—were closely correlated with the varying activity of the muscles, an explanation might be found of the variations of the use of carbohydrate which appear to occur in our experiments. We could in this way account for the fact that in our double walk experiments an increase in carbohydrate utilization occurs as soon as the second walk begins and is maintained as long as the walk lasts. Tempting though such an hypothesis may be no precision can be given to it until we have far more information as to the exact part played by insulin and other endocrine secretions in carbohydrate metabolism, but we feel that the possible influence of these secretions during muscular activity may well repay further investigation.

## SUMMARY

The effects of a long walk on the general metabolism of two subjects who were not in athletic training have been studied in detail, observations being made not only during the walk but also during a subsequent period of rest lasting 3 hours. The standard exercise in most of the experiments was a walk of 10 miles at a rate of 4·5 miles per hour.

In the post-absorptive subject who had previously been living on his normal diet it is shown that the respiratory quotient during the work rises somewhat above the preliminary resting value in the early stages and falls gradually as the work continues, and that these changes cannot be ascribed to the accumulation of excess lactic acid in the blood, to rise of body temperature, to a material degree of change in the $CO_2$ combining power of the blood, or to a developing ketosis. It is therefore concluded that the respiratory quotient under the conditions of the experiments can be taken as an index of the materials oxidized in the body, implying that during the course of the work about 100 gm of carbohydrate were used and that as the work continues there is a substantial alteration in the ratio of carbohydrate to fat metabolized.

When walking at the slower rate of 3·5 miles per hour the respiratory quotient during the walk may alter but little from that during preliminary rest.

During rest after the exercise in these post-absorptive experiments there is a fall of the respiratory quotient to the average figures of 0·76 and 0·73 in the two subjects, which is due to the fact that the oxygen consumption is higher and the $CO_2$ production either the same as, or somewhat lower than, the pre-exercise values, and this fall persists unchanged for 3 hours, and even in one experiment for as long as 9½ hours. This persistent fall in quotient was accompanied by a definite excess of ketone bodies in the blood and ketonuria, and by a progressive fall in the $CO_2$ combining power of the blood, although the alveolar $CO_2$ pressure fell only slightly, if at all, below the pre-exercise level. Only a trifling excess of lactic acid could be detected in blood samples taken just after stopping the walk; in later samples the lactic acid concentration was the same as during preliminary

rest. An abnormally low quotient shown by one of the subjects half an hour after stopping work seemed to owe its explanation rather to the fall of body temperature than to the oxidative removal of lactic acid.

Ketosis never developed during an uninterrupted 10- or even 12-mile walk, but appeared very soon after resuming rest. The onset of ketosis seemed to be associated rather with the total amount of muscular work done previously than with the rate at which that work was done. Thus walking at 3·5 miles per hour for a length of time that implied the same total oxygen consumption as a 10-mile walk at 4·5 miles per hour produced much the same after-effects as the walk at the faster rate, although the actual carbohydrate consumption during the walk appeared to be less.

In the post-absorptive subject who had taken a high carbohydrate diet on the previous day the general course of the respiratory exchange was much the same, save that the respiratory quotient ran throughout at a higher level. No ketosis developed after the exercise, nor did the progressive fall of the $CO_2$ combining power of the blood occur. Experiments of this type suggested that under normal dietetic conditions neither subject had made anything like full use of his capacity for storing glycogen, and that consequently the use of so moderate a quantity as 100 gm of carbohydrate during work under normal post-absorptive conditions might imply a really significant depletion of available carbohydrate in the body.

If, however, after taking a normal diet on the previous day, a normal breakfast was taken, or 50 gm of either glucose or fructose with 20–40 gm of bread were ingested shortly before beginning the experiment, post-exercise ketonuria developed in the majority of the experiments and the respiratory quotient fell nearly as low as in the normal post-absorptive experiments. The main result of such ingestion of sugar seemed to be so to enhance the use of carbohydrate (though some carbohydrate may possibly have been converted into stored fat) during the work that at the end of exercise the general condition was almost the same as in the ordinary post-absorptive experiments.

Confirmation has therefore been obtained of the view that a diet rich in carbohydrate taken on the day preceding the exercise may in certain respects have a greater influence than the ingestion of a quantity of easily assimilable carbohydrate shortly before beginning the work.

The previous ingestion of $NaH_2PO_4$ was not found to have any appreciable influence on the course of events in the normal post-absorptive experiments.

The non-protein respiratory quotient never fell to so low a figure during rest after exercise, save possibly on one occasion, as to afford indubitable evidence of the conversion of fat into stored carbohydrate. Reasons are given for supposing that such a conversion must have been limited in extent and insufficient to cause a material replenishment of the stores of available carbohydrate in the body. The main factor which is responsible for the persistent low quotient and ketosis appears to be the low ratio of carbohydrate to fat oxidized.

Although the stores of available carbohydrate may have been depleted at the end of a 10-mile walk they were not exhausted, since repetition of the walk for a further 6 miles at the standard rate after an interval of rest resulted in a rise of the respiratory quotient which was maintained throughout the exercise to fall abruptly again as soon as the exercise stopped.

The second walk was accompanied by no further change, or only a relatively slight increase, in the concentration of ketone bodies found in the blood during the intermediate rest period, although the ketosis became pronounced in the final rest period.

During the post-exercise rest period the blood sugar concentration never fell below the normal limits. Ingestion of sucrose, glucose, or fructose gave, however, distinct evidence of a reduced sugar tolerance. On this basis the possibility is discussed that the function of the endocrine organs associated with carbohydrate metabolism may be correlated with the varying activity of the muscles, and so afford at least a partial explanation of the changes in carbohydrate metabolism which result from muscular exercise.

## REFERENCES

Amar, J. (1910). " Le Rendement de la Machine humaine," Paris.

Benedict, F. G., and Cathcart, E. P. (1913). 'Publ. Carnegie Inst.,' No. 187.

Benedict, F. G., and Murschhauser, H. (1915). 'Publ. Carnegie Inst.,' No. 231.

Best, C. H., Hoet, J. P., and Marks, H. P. (1926). 'Proc. Roy. Soc.,' B, vol. **100**, p. 32.

Bock, A. V., Vancaulaert, C., Dill, D. B., Fölling, A., and Hurxthal, L. M. (1928). 'J. Physiol.,' vol. **66**, p. 136.

Bollman, J. L., Mann, F. C., and Magath, T. B. (1925). 'Amer. J. Physiol.,' vol. **74**, p. 238.

Campbell, J. M. H., Douglas, C. G., and Hobson, F. G. (1920). 'Phil. Trans.,' B, vol. **210**, p. 1.

Carpenter, T. M., and Fox, E. L. (1931). 'Arbeitsphysiologie,' vol. **4**, pp. 532, 570.

Cathcart, E. P., and Markowitz, J. (1927). 'J. Physiol.,' vol. **63**, p. 309.

Chaikoff, I. L. (1927). 'J. biol. Chem.,' vol. **74**, p. 203.

Christensen, E. H., Krogh, A., and Lindhard, J. (1934). 'Quart. Bull. Health Organization of the League of Nations,' vol. **3**, extract No. 13.

Cook, L. C., and Hurst, R. H. (1933). 'J. Physiol.,' vol. **79**, p. 443.

Dann, M., and Chambers, W. H. (1930). 'J. biol. Chem.,' vol. **89**, p. 675.

Denigès, C. (1898). ' C.R. Acad. Sci. Paris,' vol. **126**, p. 1868.

Dill, D. B., Edwards, H. T., and Talbott, J. H. (1933). ' J. Physiol.,' vol. **77**, p. 49.

Douglas, C. G., Haldane, J. S., Henderson, J., and Schneider, E. C. (1913). ' Phil. Trans.,' B, vol. **203**, p. 185.

Douglas, C. G., and Havard, R. E. (1932). ' J. Physiol.,' vol. **74**, p. 471.

Douglas, C. G., and Priestley, J. G. (1924). " Human Physiology," Oxford Univ. Press.

Edwards, H. T., Margaria, R., and Dill, D. B. (1934). ' Amer. J. Physiol.,' vol. **108**, p. 203.

Embden, G., Grafe, E., and Schmitz, E. (1921). ' Z. physiol. Chem.,' vol. **113**, p. 67.

Fisher, N. F., and Lackey, R. W. (1925). ' Amer. J. Physiol.,' vol. **72**, p. 43.

Friedemann, T. E., Cotonio, M., and Shaffer, P. A. (1927). ' J. biol. Chem.,' vol. **73**, p. 335.

Friedemann, T. E., and Kendall, A. I. (1929). ' J. biol. Chem.,' vol. **82**, p. 23.

Gemmill, C. L. (1934). ' Amer. J. Physiol.,' vol. **108**, p. 55.

Haldane, J. S. (1920). ' J. Path. Bact. Lond.,' vol. **23**, p. 443.

Himsworth, H. P. (1934). ' J. Physiol.,' vol. **81**, p. 29.

Junkersdorf, P. (1921, *a*). ' Pflügers Arch.,' vol. **186**, p. 238.

—— (1921, *b*). *Ibid.*, vol. **187**, p. 269.

—— (1922). *Ibid.*, vol. **197**, p. 500.

—— (1923). *Ibid.*, vol. **200**, p. 443.

—— (1925). *Ibid.*, vol. **210**, p. 351.

Krogh, A., and Lindhard, J. (1920). ' Biochem. J.,' vol. **14**, p. 290.

Lusk, G. (1928). " Science of Nutrition," Philadelphia.

Macleod, J. J. R. (1930). ' Lancet,' vol. **2**, p. 512.

Owles, W. H. (1930). ' J. Physiol.,' vol. **69**, p. 214.

Peters, J. P., and van Slyke, D. D. (1932). " Quantitative Clinical Chemistry," vol. **2**, London.

Poulton, E. P. (1933). ' Proc. R. Soc. Med.,' vol. **26**, p. 1591.

Rapport, D. (1930). ' Physiol. Rev.,' vol. **10**, p. 349.

Schöndorff, B. (1903). ' Pflügers Arch.,' vol. **99**, p. 191.

Shaffer, P. A. (1921). ' J. biol. Chem.,' vol. **47**, p. 433.

—— (1923). ' Medicine,' vol. **2**, p. 375.

Smith, H. M. (1922). ' Publ. Carnegie Instn.,' No. 309.

Staub, H. (1922). ' Z. klin. Med.,' vol. **93**, pp. 89, 123.

Sweeney, J. S. (1927). ' Arch. int. Med.,' vol. **40**, p. 818.

van Slyke, D. D. (1917). ' J. biol. Chem.,' vol. **32**, pp. 455, 495.

Woodyatt, R. T. (1921). ' Arch. int. Med.,' vol. **28**, p. 125.

612 . 816

# The Strength-Duration Relation for Electric Excitation of Medullated Nerve

By A. V. HILL, Sec. R.S., Foulerton Research Professor

(From the Department of Physiology, University College, London)

(*Received December* 2, 1935)

For condenser excitation of medullated nerve, equation (32) in the preceding paper (Hill, 1936) gave the relation between the potential E and the time-constant RC of the condenser discharge: viz.—

$$E/E_0 = (RC/k)^{\frac{1}{RC/k-1}}. \qquad (1)$$

Here $E_0$ is the rheobasic potential, $k$ the time-constant of excitation. Rushton (1931, p. 272) and H. A. Blair (1932, *b*) gave the same equation. The latter (1932, *d*) showed that it agrees well with the published results of various other workers, and later (1934) found it in equally good agreement with his own experiments. The validity of the formula, for frog's medullated nerve, has been further confirmed, down to very short times (small RC's), in experiments to be described below. It takes no account of " accommodation ". No doubt, as in the case of constant current pulses (*see* § 7 of the preceding paper), the general effect of " accommodation " will be to make the apparent value of $k$ rather too low, without causing any very obvious failure to fit the theoretical curve.

For excitation by constant current pulses, the relation (neglecting " accommodation ": equation (18) in the preceding paper) between current I and duration $x$ is,

$$I/I_0 = 1/(1 - e^{-x/k}). \qquad (2)$$

This equation was discussed by Lapicque (1907) and by various others since. It fits the facts sometimes very well (*see* below): when it does not, one may look for a cause in the reasons discussed below (p. 451).

When " accommodation " is taken into account equations (17) and (17′) of the preceding paper, for constant current pulses, are required.

*The Fitting of Strength-duration Curves, and the Determination of k*— Neglecting " accommodation ", equation (2) above for constant current pulses involves (i) the ratio of current to rheobase, *i.e.*, $I/I_0$, and (ii) the ratio of duration to time-constant of nerve, *i.e.*, $x/k$: for condenser discharges, equation (1) involves (i) the ratio of potential to rheobasic potential, *i.e.*, $E/E_0$, and (ii) the ratio of time-constant of discharge to time-constant of nerve, *i.e.*, $RC/k$. Thus, if I (or E) be plotted against $x$ (or RC), a change in rheobase, or of $k$, merely alters the scale of the curve along the appropriate axis: while if, as Rushton (1931) suggested, log I (or log E) be plotted against log $x$ (or log RC), a change in either parameter merely shifts the curve parallel to the appropriate axis, without changing its shape at all. Thus equations (1) and (2) above, if plotted in logarithmic coordinates along both axes, give curves which are invariable in size and shape.

This provides an effective means of fitting the best theoretical equation to observed points. The theoretical relation between log I (or log E) and log $x$ (or log RC) is drawn on squared paper on a sufficiently large scale. It is found best, for accuracy, as Rushton recommends, to plot the intensity coordinate on twice the scale of the time-coordinate. A copy of the theoretical curve is made on tracing cloth. The observed points are then plotted on the original, or on a similar piece of squared paper, and the tracing cloth curve is moved about, parallel to the axes, until the best fit is obtained. The position of the curve then gives the values (i) of the rheobase, and (ii) of $k$.

TABLE I

Constant current pulses: $\log_{10} I/I_0 = -\log_{10}(1 - e^{-x/k})$

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| $\mathrm{Log}_{10}\, x/k$ . . | $\bar{2}\cdot000$ | $\bar{2}\cdot301$ | $\bar{2}\cdot477$ | $\bar{2}\cdot602$ | $\bar{2}\cdot699$ | $\bar{2}\cdot778$ | $\bar{2}\cdot845$ | $\bar{1}\cdot000$ | $\bar{1}\cdot176$ |
| $\mathrm{Log}_{10}\, I/I_0$ . . | 2·002 | 1·703 | 1·529 | 1·407 | 1·311 | 1·235 | 1·170 | 1·021 | 0·856 |
| $\mathrm{Log}_{10}\, x/k$ . . | $\bar{1}\cdot301$ | $\bar{1}\cdot477$ | $\bar{1}\cdot602$ | $\bar{1}\cdot699$ | 1·778 | $\bar{1}\cdot903$ | 0·000 | 0·079 | 0·146 |
| $\mathrm{Log}_{10}\, I/I_0$ . . | 0·742 | 0·586 | 0·482 | 0·406 | 0·346 | 0·259 | 0·199 | 0·156 | 0·123 |
| $\mathrm{Log}_{10}\, x/k$ . . | 0·230 | 0·301 | 0·398 | 0·477 | 0·602 | 0·699 | 0·778 | 0·903 | |
| $\mathrm{Log}_{10}\, I/I_0$ . . | 0·088 | 0·063 | 0·037 | 0·022 | 0·008 | 0·003 | 0·001 | 0·000 | |

Condenser discharges: $\log_{10} E/E_0 = \{\log_{10} RC/k\}/(RC/k - 1)$

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| $\mathrm{Log}_{10}\, RC/k$ | $\bar{3}\cdot000$ | $\bar{3}\cdot523$ | $\bar{2}\cdot000$ | $\bar{2}\cdot255$ | $\bar{2}\cdot523$ | $\bar{2}\cdot740$ | $\bar{1}\cdot000$ | $\bar{1}\cdot301$ | $\bar{1}\cdot544$ |
| $\mathrm{Log}_{10}\, E/E_r$ | 3·003 | 2·486 | 2·020 | 1·775 | 1·527 | 1·334 | 1·111 | 0·874 | 0·701 |
| $\mathrm{Log}_{10}\, RC/k$ | $\bar{1}\cdot699$ | $\bar{1}\cdot824$ | 0·000 | 0·176 | 0·301 | 0·398 | 0·544 | 0·699 | 0·845 |
| $\mathrm{Log}_{10}\, E/E_r$ | 0·602 | 0·529 | 0·434 | 0·356 | 0·301 | 0·265 | 0·217 | 0·175 | 0·141 |
| $\mathrm{Log}_{10}\, RC/k$ | 1·000 | 1·204 | 1·477 | 1·699 | 2·000 | 2·477 | 3·000 | | |
| $\mathrm{Log}_{10}\, E/E_r$ | 0·111 | 0·080 | 0·051 | 0·034 | 0·020 | 0·009 | 0·003 | | |

Table I may be used in constructing the curves, which are shown on a small scale in fig. 1. They have the following characteristics:—

(*a*) They both converge upwards and to the left to the same asymptote at a slope of $-1$ ($-2$ as plotted). This asymptote corresponds to a constant *quantity* in the electric discharge required for excitation.

(*b*) They both converge to the right to the same horizontal asymptote, they both indicate the existence of a finite rheobase.

(*c*) The duration for double the rheobase ($\log I = \log I_0 + 0{\cdot}301$, or $\log E = \log E_0 + 0{\cdot}301$), given at points D, is

| | |
|---|---|
| constant current pulses ................ | $x = 0{\cdot}693k$ |
| condenser discharges .................. | $RC = 2k$. |

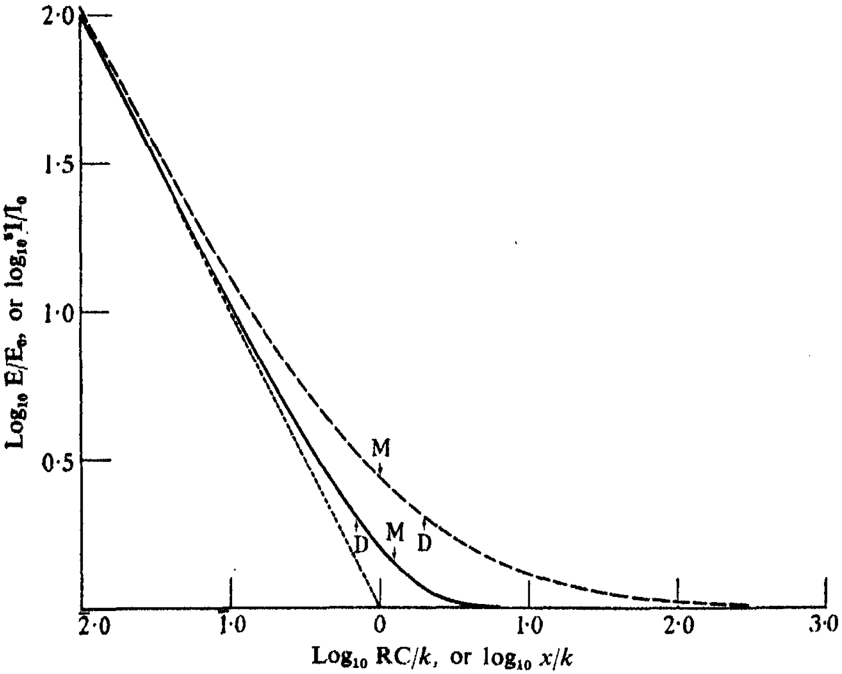


FIG. 1—Strength-duration curves, in double logarithmic coordinates, for condenser discharges and for constant current pulses, calculated respectively from equations (1) and (2). Note that $\log E/E_0$ (or $\log I/I_0$) is plotted on twice the scale of $\log RC/k$ (or $\log x/k$). The slope of the asymptote to the left is $-1$ ($-2$ as plotted). The slope of the tangent at the point M of minimum energy is $-\frac{1}{2}$ ($-1$ as plotted). D is the point at which the stimulus is double the rheobase. - - - condenser discharges;—— constant current pulses.

The ratio $x/RC = 0{\cdot}347$. Lapicque (1926, p. 324) gives the experimental value $0{\cdot}37$. The agreement is good.

(*d*) The curve for condenser discharges falls much less rapidly than the curve for constant current pulses. This is because the " utilization time " of a condenser discharge becomes progressively a smaller fraction of RC as RC is increased.

(*e*) The point M of minimum energy (slope of tangent $-\frac{1}{2}, -1$ as plotted) on the curve for condenser discharges, is exactly above the intersection of the asymptotes, at the point for which $RC = k$. This provides a method of determining $k$. When $RC = k$, $E/E_0 = e = 2\cdot72$. For constant current pulses, minimum energy is for $x/k = 1\cdot26$, for which $I/I_0 = 1\cdot40$.

## EXPERIMENTAL

Experiments have been made with condenser discharges on the sciatic nerves of Hungarian *Rana esculenta*. The agreement between the calculated curve and the experimental points is usually very good. This was shown graphically in fig. 1 of a previous note (Hill, 1935), but can be better expressed numerically as in Table II, where log E, calculated and observed, can be directly compared. The mean difference is just less than 0·01, which represents an average divergence between E, calculated and observed, of only 2%. The range investigated was a wide one: 4600, 1600, 1100, and 3100-fold respectively, in regard to RC, in the experiments quoted.

The method used has been described in general in various places (Scott, 1934; Hill, 1934; Bugnard and Hill, 1935). It employs repetitive stimulation by condenser and commutator, adjusted by varying E for each RC to give a constant (submaximal) monophasic electric response of the nerve, as an index of constant excitation. The wide range of RC is obtained, not by varying C alone, which would mean going to very low values for short times—when the capacity of the circuit would not be negligible—but by using a series of 3 or 4 different shunts with which the whole range is reached with reasonable values of C. The value of R, the total discharge resistance with any shunt, is measured in each experiment with a bridge suitably connected to the circuit, employing a weak direct current and a sensitive galvanometer: there is no difficulty about this if the electrodes are properly non-polarizable. The same value of RC can be set in different ways, *e.g.*, low R and high C, or high R and low C. Experiment has confirmed the theoretical expectation that the nerve cannot distinguish between different methods of making up the same value of RC. A large resistance was always used in series with the nerve, so as to swamp any variations of resistance in that.

Non-polarizable electrodes were employed, consisting of calomel half-cells and fluid connexions to the nerve. The contacts were at first of filter or drawing paper soaked in Ringer's solution: later, thin strips of soft wood, which can be shaped as required, have been preferred: these are kept in Ringer's solution till needed, they can be placed in position n a few seconds in pools of solution to which the calomel half-cells are connected, and they are convenient and accurate to use. The calomel cells should be watched and kept supplied with sufficient calomel, or they may cease to be non-polarizable.

*Experiment of November* 24, 1934 (the results of which are given in Table II)—Two sciatic nerves of Hungarian frog in aerated Ringer's solution for ½ hour, then in parallel on electrodes in paraffin chamber at 4° C. The electrodes were of soft wood, with rather sharp edges, saturated by long soaking in Ringer's solution, and connected to calomel half-cells. Resistance of nerve circuit (nerve + electrodes + series resistances) 125,000 ohms, shunted by various resistances to give the resultant discharge resistance R shown in first row: R was measured directly with a bridge. The electrodes were, in order, $S_1$ anode, $S_2$ cathode, of stimulating current, $G_1$ and $G_2$ galvanometer leads, $G_2$ being on an injured point of the nerve: $S_1S_2 = 1{\cdot}2$ cm, $S_2G_1 = 2{\cdot}0$ cm, $G_1G_2 = 2{\cdot}2$ cm. Five one-way shocks per second with commutator and condenser system. Maximal response on galvanometer scale about 50 mm: the response taken as index of constant excitation was 10 mm. The observations were made with diminishing RC and repeated in the reverse order: the mean value of E was taken for each RC. Identical values within ± 2% were obtained in separate series, for a response 20% of maximal, at 12 shocks per second and at 21 shocks per second. The rheobase $E_0$ (the first column) was obtained for constant current pulses of duration about 0·08 second at the frequency of 5 per second. The calculated results are for $\log k = \bar{3}{\cdot}04$ ($k = 1100$ μ sec) and $\log E_0 = \bar{1}{\cdot}395$. The observations and a theoretical curve are given in fig. 1 of a previous note (Hill, 1935).

TABLE II

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| R: ohms .... | | 57000 | 17200 | 17200 | 17200 | 17200 | 17200 | 17200 |
| C: farads .... | | $1{\cdot}1 \times 10^{-6}$ | 1·1 | 0·4 | 0·15 | 0·10 | 0·05 | 0·035 |
| Log RC .... | | $\bar{2}{\cdot}795$ | $\bar{2}{\cdot}275$ | $\bar{3}{\cdot}840$ | $\bar{3}{\cdot}410$ | $\bar{3}{\cdot}235$ | $\bar{4}{\cdot}935$ | $\bar{4}{\cdot}780$ |
| Log E, obs. .. | $\bar{1}{\cdot}395$ | $\bar{1}{\cdot}420$ | $\bar{1}{\cdot}475$ | $\bar{1}{\cdot}555$ | $\bar{1}{\cdot}680$ | $\bar{1}{\cdot}750$ | $\bar{1}{\cdot}895$ | $\bar{1}{\cdot}975$ |
| Log E, calc. | $\bar{1}{\cdot}395$ | $\bar{1}{\cdot}425$ | $\bar{1}{\cdot}470$ | $\bar{1}{\cdot}545$ | $\bar{1}{\cdot}670$ | $\bar{1}{\cdot}740$ | $\bar{1}{\cdot}885$ | $\bar{1}{\cdot}975$ |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| R: ohms .... | 17200 | 1001 | 1001 | 1001 | 1001 | 171·5 | 171·5 |
| C: farads .... | $0{\cdot}020 \times 10^{-6}$ | 0·34 | 0·15 | 0·05 | 0·02 | 0·119 | 0·08 |
| Log RC .... | $\bar{4}{\cdot}535$ | $\bar{4}{\cdot}530$ | $\bar{4}{\cdot}175$ | $\bar{5}{\cdot}700$ | $\bar{5}{\cdot}300$ | $\bar{5}{\cdot}310$ | $\bar{5}{\cdot}135$ |
| Log E, obs. .. | 0·130 | 0·130 | 0·395 | 0·810 | 1·185 | 1·185 | 1·360 |
| Log E, calc. | 0·130 | 0·135 | 0·400 | 0·800 | 1·160 | 1·155 | 1·320 |

*Experiment of November* 28, 1934 (Table III)—Two sciatic nerves of Hungarian frog, in aerated Ringer's solution 1½ hours, then on Ringer-saturated wooden electrodes connected to calomel half-cells: first series at 22·5° C: then chamber and nerve in cold store for 2¼ hours, then second series at 6·2° C. Two-way shocks to avoid electrotonic leaks, otherwise apparent in this experiment; at 22·5° C, 2 × 15·7 shocks per second; at 6·2° C, 2 × 8·6 shocks per second. Resistance of nerve circuit, 43,500 ohms (22·5° C), 51,500 ohms (6·2° C), shunted by various resistances to give the resultant discharge resistance R (directly measured) shown in the first row. At each temperature, series, and reverse. Calculated results; 22·5° C, for log $k = \bar{4}\cdot53$, log $E_0 = \bar{1}\cdot85$; 6·2° C, for log $k = \bar{4}\cdot95$, log $E_0 = \bar{1}\cdot54$.

TABLE III

22·5° C

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| R: ohms .... | 13550 | 13500 | 13550 | 13550 | 13500 | 13500 | 983 | 983 |
| C: farads .... | $1\cdot0 \times 10^{-6}$ | 0·52 | 0·33 | 0·20 | 0·10 | 0·05 | 1·0 | 0·5 |
| Log RC .... | $\bar{2}\cdot13$ | $\bar{3}\cdot85$ | $\bar{3}\cdot65$ | $\bar{3}\cdot43$ | $\bar{3}\cdot13$ | $\bar{4}\cdot83$ | $\bar{4}\cdot99$ | $\bar{4}\cdot69$ |
| Log E, obs. .. | $\bar{1}\cdot89$ | $\bar{1}\cdot92$ | $\bar{1}\cdot94$ | $\bar{1}\cdot98$ | 0·05 | 0·15 | 0·10 | 0·21 |
| Log E, calc. | $\bar{1}\cdot94$ | $\bar{1}\cdot92$ | $\bar{1}\cdot94$ | $\bar{1}\cdot98$ | 0·04 | 0·14 | 0·09 | 0·21 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| R: ohms .... | 983 | 983 | 983 | 983 | 170·2 | 170·2 | 170·2 | 170·2 |
| C: farads .... | $0\cdot33 \times 10^{-6}$ | 0·20 | 0·11 | 0·06 | 0·30 | 0·20 | 0·10 | 0·05 |
| Log RC .... | $\bar{4}\cdot51$ | $\bar{4}\cdot29$ | $\bar{4}\cdot03$ | $\bar{5}\cdot77$ | $\bar{5}\cdot71$ | $\bar{5}\cdot53$ | $\bar{5}\cdot23$ | $\bar{6}\cdot93$ |
| Log E, obs. .. | 0·29 | 0·42 | 0·58 | 0·77 | 0·82 | 0·96 | 1·22 | 1·49 |
| Log E, calc. | 0·29 | 0·41 | 0·57 | 0·73 | 0·83 | 0·96 | 1·22 | 1·49 |

6·2° C

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| R: ohms .... | 14400 | 14400 | 14400 | 14400 | 14400 | 14400 | 987 | 987 |
| C: farads .... | $1\cdot0 \times 10^{-6}$ | 0·6 | 0·3 | 0·18 | 0·10 | 0·05 | 0·5 | 0·3 |
| Log RC...... | $\bar{2}\cdot16$ | $\bar{3}\cdot94$ | $\bar{3}\cdot63$ | $\bar{3}\cdot41$ | $\bar{3}\cdot16$ | $\bar{4}\cdot86$ | $\bar{4}\cdot69$ | $\bar{4}\cdot47$ |
| Log E, obs. .. | $\bar{1}\cdot62$ | $\bar{1}\cdot65$ | $\bar{1}\cdot72$ | $\bar{1}\cdot78$ | $\bar{1}\cdot87$ | 0·02 | 0·10 | 0·24 |
| Log E, calc. | $\bar{1}\cdot62$ | $\bar{1}\cdot65$ | $\bar{1}\cdot72$ | $\bar{1}\cdot78$ | $\bar{1}\cdot88$ | 0·02 | 0·12 | 0·26 |

| | | | | | |
|---|---|---|---|---|---|
| R: ohms .... | 987 | 987 | 170·2 | 170·2 | 170·2 |
| C: farads .... | $0\cdot15 \times 10^{-6}$ | 0·08 | 0·3 | 0·15 | 0·08 |
| Log RC .... | $\bar{4}\cdot15$ | $\bar{5}\cdot90$ | $\bar{5}\cdot71$ | $\bar{5}\cdot41$ | $\bar{5}\cdot13$ |
| Log E, obs. .. | 0·45 | 0·68 | 0·84 | 1·12 | 1·38 |
| Log E, calc. | 0·49 | 0·69 | 0·86 | 1·13 | 1·39 |

*Experiment of December* 18, 1934 (Table IV)—Two sciatic nerves of Hungarian frog at 20° C in parallel on Ringer-saturated wooden electrodes connected to calomel half-cells. Resistance of nerve circuit about 40,000 ohms, shunted by various resistances to give the resultant discharge resistance R (directly measured) shown in the first row. Thirty one-way shocks per second, response taken as index of constant excitation nearly minimal, viz., 3 mm on galvanometer scale. Series and reverse. Calculated results for, log $k = \bar{4}\cdot 73$ ($k = 537\ \mu$ sec), log $E_0 = \bar{1}\cdot 36$.

TABLE IV

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| R : ohms ...... | 38700 | 15050 | 15050 | 15050 | 15050 | 15050 | 995 |
| C : farads ...... | $1\cdot 1 \times 10^{-6}$ | 1·1 | 0·5 | 0·25 | 0·15 | 0·08 | 0·5 |
| Log RC ...... | $\bar{2}\cdot 63$ | $\bar{2}\cdot 22$ | $\bar{3}\cdot 88$ | $\bar{3}\cdot 58$ | $\bar{3}\cdot 35$ | $\bar{3}\cdot 08$ | $\bar{4}\cdot 70$ |
| Log E, obs. .... | $\bar{1}\cdot 39$ | $\bar{1}\cdot 42$ | $\bar{1}\cdot 45$ | $\bar{1}\cdot 49$ | $\bar{1}\cdot 55$ | $\bar{1}\cdot 64$ | $\bar{1}\cdot 80$ |
| Log E, calc. .. | $\bar{1}\cdot 39$ | $\bar{1}\cdot 41$ | $\bar{1}\cdot 45$ | $\bar{1}\cdot 50$ | $\bar{1}\cdot 55$ | $\bar{1}\cdot 64$ | $\bar{1}\cdot 81$ |

| | | | | | | |
|---|---|---|---|---|---|---|
| R : ohms ...... | 995 | 995 | 995 | 171·2 | 171·2 | 171·2 |
| C : farads ...... | $0\cdot 25 \times 10^{-6}$ | 0·15 | 0·08 | 0·25 | 0·15 | 0·08 |
| Log RC ...... | $\bar{4}\cdot 40$ | $\bar{4}\cdot 17$ | $\bar{5}\cdot 90$ | $\bar{5}\cdot 63$ | $\bar{5}\cdot 41$ | $\bar{5}\cdot 14$ |
| Log E, obs. .... | $\bar{1}\cdot 97$ | 0·12 | 0·34 | 0·56 | 0·75 | 1·00 |
| Log E, calc. .. | $\bar{1}\cdot 98$ | 0·13 | 0·34 | 0·55 | 0·75 | 0·99 |

The only error of any importance which affects the method, apart from ordinary electric leaks, is the escape of electrotonic current to the galvanometer. If a current be passed through a nerve between $S_1$ and $S_2$ (the stimulating electrodes), an electrotonic current will flow in a galvanometer connected to $G_1$ and $G_2$, even though $G_1$ and $G_2$ are external to $S_1$ $S_2$. It depends upon the distance of the G's from the S's, and upon the strength of the polarizing current. It will occur just the same if the latter is made up of intermittent one-way discharges. The only region of the intensity-duration curve in which this error is liable to occur is at long times, *i.e.*, with large quantities of electricity, and for that reason a direct determination of $E_0$ with very long condenser discharges has frequently been avoided. The error can be detected by using subthreshold discharges; if a small deflexion occurs which is reversed by reversing the current, then it is due to electrotonic leak. In that case the best way to avoid trouble is to use two-way instead of one-way stimulation, for then the effect of electrotonic leak is altogether eliminated (*see* the experiment of November 28).

Experiments have been performed for various purposes, in which $k$, the time-constant of the intensity-duration curve, has been measured with electrodes 15 mm or more apart. On the sciatic nerves of Hungarian frogs, at 4–6° C, $k$ is usually 800 to 1100 $\mu$ sec: at 19–23° C, 300 to 500 $\mu$ sec. The corresponding chronaxie, being $0\cdot 693k$, is 0·55 to 0·75 m sec at the lower temperature, 0·2 to 0·35 m sec at the higher. In these experiments between 4–6° C and 19–23° C the temperature coefficient of $1/k$ was found to be $Q_{10} = 1\cdot 8$, the same as Lucas found (1908) for the velocity of propagation of an impulse in nerve. Lapicque (1926, footnote, p. 232) gives a $Q_{10}$ of 1·7 for the chronaxie of nerve at an "ordinary temperature", of 2·0 and above at lower temperatures (*see also* Lapicque, 1934, p. 116; Lucas and Mines, 1907; Rushton, 1932, referred to by Hill, 1932, p. 62). Blair (1935), from an analysis of the older data, together with some new data of his own, concluded that $Q_{10}$ for $1/k$ over the lower range of temperature (below 20° C) is about 2·0, but in the upper range considerably less. Table III refers to a few special experiments.

TABLE V

*November* 21 : 4° C—Nerve dissected to peroneal and tibial branches.
Stimulated at peripheral end, recorded centrally, $k = 970$ μ sec.
Stimulated at central end, recorded peripherally, $k = 795$ μ sec.
Stimulated at central end, during continuous maximal stimulation at 16 shocks per second, the extra test shocks at 16 per second being exactly halfway between those of the primary series, $k = 600$ μ sec.

*November* 24 : 4° C—Ordinary electrodes, $k = 1100$ μ sec.
Electrodes for transverse excitation (two wooden edges opposite one another) required 3½ times $E_0$ but gave the identical value of $k$.

*November* 28 : 22½° C—$k = 340$ μ sec.
6·2° C—3 hours later, $k = 890$ μ sec.
$Q_{10}$ for $1/k = 1·8$.
Same nerve at 4·7° C, 15 hours later, $k = 1100$ μ sec.

*November* 29 :
6° C—12 h. 15 m.–12 h. 45 m. : $k = 760$ μ sec.
14 h. 10 m.–14 h. 55 m. : $k = 810$ μ sec.
20·5° C—16 h. 30 m.–17 h. 10 m. : $k = 330$ μ sec.
21·1° C—17 h. 15 m.–18 h. 0 m. : $k = 350$ μ sec.
$Q_{10}$ for $1/k = 1·8$.

*December* 3 :
19·1° C—13 h. 0 m.–14 h. 0 m. : $k = 360$ μ sec.
5·2° C—16 h. 30 m.–17 h. 20 m. : $k = 795$ μ sec.
Q/10 for $1/k = 1·8$.

During a visit to this laboratory in January and February, 1935, Professor J. P. Bouckaert made a number of experiments (*a*) on the effect of inter-electrode distance on the time-constant $k$; and (*b*) on the effect of using a small reading, as index of constant response, on the form of the log E — log RC curve.

These are referred to in abstract by Bouckaert and Katz (1935), and Professor Bouckaert has kindly allowed me to give further details here.

(*a*) *Inter-electrode Distance*—Sharp-edged soft wood electrodes impregnated with Ringer's fluid were used, the distance being varied between 0·5 and 20 mm. Stimulation was at the peripheral ends of the tibial and

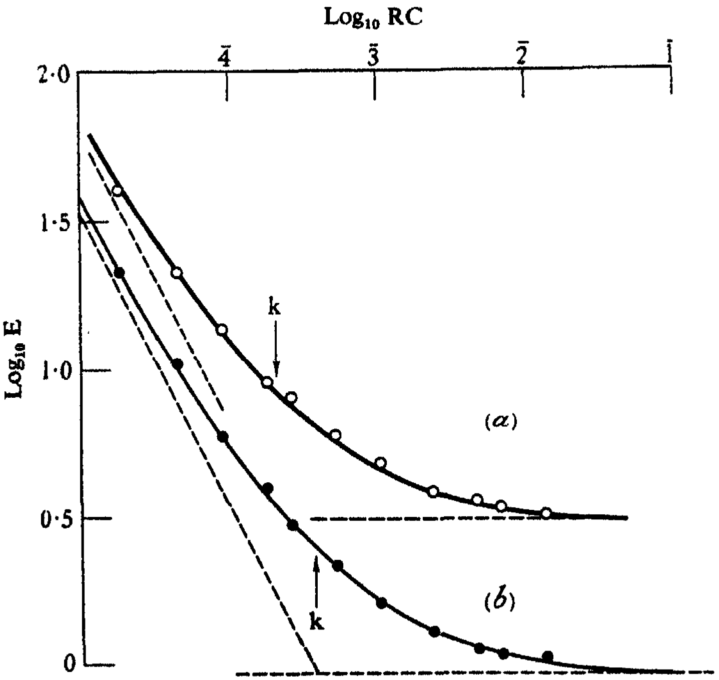


FIG. 2—February 2, 1935—Sciatic of *Rana esculenta* at 20·8° C. Low-frequency repetitive stimulation. Index of response, semi-maximal "negative variation". Electrodes $S_1$ $S_2$ $S_3$ $G_1$ $G_2$ in order: $S_1 S_2 = 20$ mm, $S_2 S_3 < 1$ mm, $S_3 G_1 = 39$ mm, $G_1 G_2 = 15$ mm. $S_1$ and $S_2$ alternative stimulating anodes: $S_3$ stimulating cathode. $G_1$ "uninjured", $G_2$ "injured", galvanometer leads.

Strength-duration (voltage-capacity) curves calculated from equation (1) with observed points, for:—

(*a*) $< 1$ mm inter-electrode distance: $E_0 = 3 \cdot 1$ volts: $k = 204$ $\mu$ sec.
(*b*) 21 mm inter-electrode distance: $E_0 = 0 \cdot 91$ volt, $k = 372$ $\mu$ sec.

The arrows show the values of $k$, the broken lines the asymptotes of the curves.

*Note*—In this, as in all the other experiments of this type, at each condenser setting the value of E was determined for $S_2$ $S_3$ and for $S_1$ $S_3$: after a series with decreasing capacities another was made with increasing capacities, and a mean was taken of the two readings at each setting.

peroneal branches of the sciatic, where the nerve is thin. Provided that a fairly large galvanometer response is taken as index there is good agreement between observation and calculated curve, as shown in fig. 2. The rheobase is always greater for the short distance (*cf.* Rushton, 1934) and the time-constant is less. (The resistance R was separately measured, with each shunt, for long and for short distance.) Slow repetitive stimulation was employed.

Details of a single experiment (No. 4) are shown in fig. 2 and its legend: the results of six experiments are given in Table VI.

TABLE VI

| Experiment No. | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Temperature, ° C | 19 | 21 | 21·8 | 20·8 | 22 | 21 |
| Electrode distance: mm | 1 | 0·5 | $<1$ | $<1$ | $<1$ | 0·5 |
| $k$: $\mu$ sec | 204 | 145 | 204 | 204 | 123 | 170 |
| Electrode distance: mm | 15 | 20 | 20 | 21 | 15 | 20 |
| $k$: $\mu$ sec | 380 | 380 | 363 | 372 | 269 | 324 |

Thus reducing the electrode distance from 15–20 mm to less than 1 mm reduces the value of $k$ to about one-half. The effect is, of course, well known. Cardot and Laugier (1914) discovered it, Lullies (1930) and Labes and Lullies (1932) discussed it in relation to polarization, and Grundfest (1932) found very short chronaxies (down to 20 $\mu$ sec) with capillary electrodes, with which, as Lapicque points out (1934, p. 122) the effective inter-electrode distance is very small. Bouckaert's experiments reported here confirm the effect of inter-electrode distance, using non-polarizable electrodes, and show that with a distance as small as 0·5 mm the theoretical equation is just as well obeyed as with a longer distance. There is no experimental reason, therefore, why one $k$ should be regarded as " truer " than another, or why $k$, observed with a short inter-electrode distance, should be labelled " false "; though perhaps it is natural, with Lapicque (1934), to regard the constant value reached at great inter-electrode distances as a more convenient index of the time-factor in excitation.

(*b*) The effect of using a small reading as index of constant response is frequently to give a double curve as shown in fig. 3. It appears that two groups of fibres are involved, one quicker than the other. If the slower fibres are present in very limited number, increasing the stimulus to get a larger index response would raise the log E — log RC curve above the broken line over the whole range, and a single curve would result. The discontinuity shown in fig. 3 might not have been detected, had one not attempted to fit a theoretical curve to the points; in themselves the latter follow a comparatively smooth curve, which, however, does not obey equation (1).

## CONSTANT CURRENT PULSES

In fig. 1 the two curves converge to the left to the same asymptote of "constant-quantity": as Fabre, Quesnoy, and Berteaux (1934) showed experimentally that they should. H. A. Blair in several papers (1932, *a*, *c*, 1934) has advocated the use, for constant current pulses, of the equation (using the present symbols)

$$I/I_0 = 1/(1 - \beta e^{-t/k}) \qquad (3)$$

where $\beta$ is a constant the physical meaning of which is not very clear. Equation (3) does not give the constant-quantity relation at very short times, as is easily shown by expanding $e^{-t/k}$ in powers of $t$. Equation (3) is actually the same as that which I gave in 1910, but the latter was admittedly an approximation for longer times only, and the full equation for short times does obey the constant-quantity rule. As a matter of fact, in most of the experiments quoted by Blair, and particularly in his own, his value of $\beta$ is very nearly unity, and indeed just as good a fit is obtained in Blair's experiments with $\beta = 1$ precisely as with the values he gives. The following four examples (Table VII) from Table II, p. 761 of Blair's paper (1934) illustrate this. The equation tested is $V/R = 1/(1 - e^{-x/k})$ where V is threshold voltage for duration $x$, and R is rheobasic voltage.

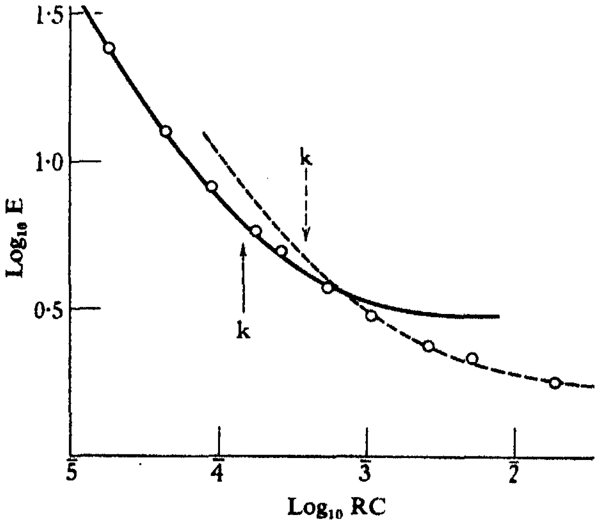


FIG. 3—January 31, 1935. Sciatic of *Rana esculenta* at 21° C. Low-frequency repetitive stimulation. Index of response, 3 mm deflexion: maximal response = 60 mm. Distance between stimulating electrodes <1 mm. Curves drawn from equation (1) with $k$'s 380 μ sec and 145 μ sec respectively.

The differences of the observed from Blair's calculated results also are given for comparison.

TABLE VII

*Experiment* 1—$k = 0\cdot402$ m sec, R = 151.

| $x$: m sec | ∞ | 2 | 1·5 | 1·25 | 1 | 0·8 | 0·6 | 0·4 | 0·35 | 0·3 | 0·26 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| V, calc. | 151 | 152 | 155 | 158 | 164½ | 175 | 195 | 239 | 260 | 287 | 316 |
| V, obs. | 151 | 151 | 155 | 161 | 169 | 176 | 199 | 237 | 255 | 282 | 317 |
| Difference | 0 | −1 | 0 | 3 | 4½ | 1 | 4 | −2 | −5 | −5 | 1 |
| Blair's difference | 0 | −1 | 0 | 2 | 2 | −1 | −1 | −4 | −4 | −2 | 7 |

*Experiment* 2—In this experiment Blair took $\beta = 1$, $k = 0\cdot320$ m sec, R = 198.

| $x$: m sec | ∞ | 2 | 1·5 | 1·25 | 1·0 | 0·8 | 0·6 | 0·4 | 0·3 | 0·26 |
|---|---|---|---|---|---|---|---|---|---|---|
| V, calc. | 198 | 198 | 199 | 202 | 207 | 216 | 233 | 277 | 324 | 354 |
| V, obs. | 198 | 200 | 203 | 204 | 210 | 213 | 239 | 275 | 317 | 368 |
| Difference | 0 | 2 | 4 | 2 | 3 | −3 | −6 | −2 | −7 | +14 |

*Experiment* 3—$k = 0\cdot334$ m sec, R = 101.

| $x$: m sec | ∞ | 2 | 1·5 | 1·25 | 1·0 | 0·8 | 0·6 | 0·4 | 0·35 | 0·30 | 0·26 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| V, calc. | 101 | 101 | 102 | 103½ | 106½ | 112 | 122½ | 147 | 156 | 170 | 186 |
| V, obs. | 101½ | 100 | 100 | 103 | 109 | 115 | 123 | 146 | 157 | 171 | 187 |
| Difference | ½ | −1 | −2 | −½ | 2½ | 3 | ½ | −1 | 1 | 1 | 1 |
| Blair's difference | 1½ | 0 | −1 | −1 | 3 | 4 | 1 | 0 | 1 | 0 | 0 |

*Experiment* 4—$k = 0\cdot575$ m sec, R = 110.

| $x$: m sec | ∞ | 2 | 1·5 | 1·25 | 1·0 | 0·8 | 0·6 | 0·4 | 0·3 | 0·26 |
|---|---|---|---|---|---|---|---|---|---|---|
| V, calc. | 110 | 113½ | 119 | 124 | 133½ | 146½ | 170 | 219 | 270 | 302 |
| V, obs. | 111½ | 110 | 113 | 120 | 143 | 159 | 174 | 222 | 268 | 300 |
| Difference | 1½ | −3½ | −6 | −4 | 9½ | 12½ | 4 | 3 | −2 | −2 |
| Blair's difference | 0 | −5 | −8 | −7 | 6 | 9 | 0 | 0 | −3 | 2 |

The accuracy of fit with $\beta = 1$ is obviously as good as with Blair's formula, so the introduction of $\beta$ is unnecessary. Four very careful experiments by Rushton (1932, p. 436), two at room temperature, two near 0° C, also give good agreement with equation (2), as shown by Hill (1932, p. 61). Such agreement is not always found, but in view of the high accuracy with which observations with condenser discharges fit equation (1) it is likely that those with constant current pulses would fit equation (2) equally well, were it possible to make them with the same precision.

The probable causes of deviation from equation (2) are:—

(i) the experimental difficulty of providing constant current pulses of exactly known duration;

(ii) "accommodation" as discussed in § 7 of the preceding paper;

(iii) the stimulation of different fibres at different parts of the curve (*cf.* fig. 3);

(iv) the impossibility, with many nerves (*e.g.*, of crabs), of avoiding repetitive response to the longer-lasting currents, and the fact that many neuro-muscular junctions do not transmit a single impulse.

The general conclusion to be drawn, therefore, is that on medullated nerve, at least, and with currents of short duration involving no change of threshold (" accommodation "), the more carefully the measurements are made the more closely do they conform to the view that the time-constant of excitation is simply that of the exponential decay of the " excitatory disturbance " produced by the current.

I am greatly indebted to Professor J. P. Bouckaert for allowing me to report his observations, and to Dr. D. Y. Solandt for frequent and ready assistance in the experiments.

## SUMMARY

A method is described of fitting the theoretical strength-duration (voltage-capacity) curve, for electric excitation of nerve, to experimental observations.

Experiments are reported with condenser discharges, over a wide range of discharge-times, confirming the theoretical relation, and the fact that the time-constant of excitation (but not the form of the curve) is considerably affected by inter-electrode distance.

With a very small electric response as index of constant excitation, the voltage-capacity curve is sometimes double, probably owing to excitation of different fibres at different durations.

The strength-duration curve for constant current pulses is discussed. Equation (2) fits certain experimental results of Blair and of Rushton well.

## REFERENCES

Blair, H. A. (1932, *a*). 'J. gen. Physiol.,' vol. **15**, p. 709.
—— (1932, *b*). *Ibid.*, vol. **15**, p. 731.
—— (1932, *c*). *Ibid.*, vol. **16**, p. 165.
—— (1932, *d*). *Ibid.*, vol. **16**, p. 177.
—— (1934). *Ibid.*, vol. **18**, p. 755.
—— (1935). 'J. cell. comp. Physiol.,' vol. **6**, p. 291.
Bouckaert, J. P., and Katz, B. (1935). 'J. Physiol.,' vol. **85**, p. 10P.
Bugnard, L., and Hill, A. V. (1935). *Ibid.*, vol. **83**, pp. 383, 394, 416, 425.
Cardot, H., and Laugier, H. (1914). 'C.R. Soc. Biol., Paris,' vol. **77**, p. 276.

Fabre P., Quesnoy, P., and Berteaux, J. (1934). *Ibid.*, vol. **116**, p. 179.
Grundfest, H. (1932). 'J. Physiol.,' vol. **76**, p. 95.
Hill, A. V. (1910). *Ibid.*, vol. **40**, p. 190.
—— (1932). "Chemical Wave Transmission in Nerve," Camb. Univ. Press.
—— (1934). 'J. Physiol.,' vol. **82**, p. 423.
—— (1935). *Ibid.*, vol. **88**, p. 30P.
—— (1936). 'Proc. Roy. Soc.,' B., vol. **119**, p. 305.
Labes, R., and Lullies, H. (1932). 'Z. Biol.,' vol. **93**, p. 211.
Lapicque, L. (1907). 'J. Physiol., Path. gén.,' vol. **9**, p. 622.
—— (1926). "L'excitabilité en fonction du temps," Press. Univ., Paris.
—— (1934). 'J. Physiol.,' vol. **81**, p. 113.
Lucas, K. (1908). *Ibid.*, vol. **37**, p. 112.
Lucas, K., and Mines, G. R. (1907). *Ibid.*, vol. **36**, p. 334.
Lullies, H. (1930). 'Pflügers Arch.,' vol. **225**, p. 69.
Rushton, W. A. H. (1931). 'J. Physiol.,' vol. **72**, p. 265.
—— (1932). *Ibid.*, vol. **74**, p. 424.
—— (1934). *Ibid.*, vol. **82**, p. 332.
Scott, D. (1934). *Ibid.*, vol. **82**, p. 321.

---

581.12

# Analytic Studies in Plant Respiration

## VI—The Relation of the Respiration of Potatoes to the Concentration of Sugars and to the Accumulation of a Depressant at Low Temperatures

## Part 3—The Relation of the Respiration to the Concentration of Sucrose

By John Barker, The Low Temperature Research Station, Cambridge

(*Communicated by F. F. Blackman, F.R.S.—Received October* 15, 1935)

### 1—Introduction

In Parts 1 and 2 of this investigation (Barker, 1933), an analysis was presented of the relation between the rate of respiration of potatoes and the concentration of extractable sugar, the latter being varied by changes in the temperature of storage over the range −1° C to +15° C. The relation between respiration and sugar was found to be complicated by a new type of temperature effect, which was called "*low temperature depression*". When the *depression* was believed to be negligible the respiration/sugar relation—*i.e.*, the relation between the rate of respiration plotted as ordinate axis and concentration of sugar as abscissa axis—showed a general approximation to the form of an " enzymatic rectangular hyperbola " such as expresses the rate of reaction/substrate concentration relation for an enzyme reaction *in vitro*, suggesting that the sugar concentration in some way determined the concentration of the respirable substrate.

From the data then available it could not be decided whether respiration were more closely related to sucrose or to one of the hexose sugars, and, pending a more precise analysis the concentration of total sugars was provisionally adopted as the measure of the respirable substrate.

In the present study attention has been focussed on certain temperature transitions which produce marked divergencies in the relative proportions of sucrose and the hexose sugars, and it has been possible to obtain not only rising sucrose with falling hexose but also the reverse changes. Under these conditions the respiration is found to be correlated with the drifts of sucrose and shows no direct relation to the changes of the hexose sugars, glucose, and fructose.

## 2—METHODS

Two stocks of small King Edward potatoes were used, experiment 1 being made in 1930 with one stock and experiments 2, 3, and 4 in 1933 with another stock. The samples for respiration and sugar determinations consisted of from 18–20 potatoes in experiments 1 and 2 and of 36–38 potatoes in experiments 3 and 4.

The output of carbon dioxide was used as the measure of the respiration; earlier experiments, in which the oxygen uptake was also determined, had shown that even at high respiration rates the respiratory quotient was unity.

The methods adopted for determining the respiration, and for preparing and clearing the sugar extracts have been described elsewhere (Hanes and Barker, 1931). The carbon dioxide production is stated as milligrams per hour per 100 grams initial fresh weight of potato tissue.

The estimation of the sugars sucrose, glucose, and fructose in the cleared extract was carried out by the micro-method of Shaffer and Hartman, as modified by Dr. E. J. Maskell.*

The reducing power was determined both before and after hydrolysis of sucrose which was effected by heating at 60° C for 25 minutes in 0·5 N hydrochloric acid. Tests with a number of samples from several stocks of potatoes showed that the increase in reducing value produced by this hydrolysis consisted in nearly equal amounts of glucose and fructose determined as described below. The increase in reducing value on hydrolysis was accordingly accepted as equivalent to the amount of sucrose and was expressed as invert sugar in percentage of fresh weight.

---

* I am deeply indebted to Dr. Maskell not only for giving me full details of this unpublished sugar method but also for much helpful advice as regards the technique of sugar estimation and the analysis of the results recorded in this paper.

Glucose could not be determined by the direct estimation of the iodine reduction of the extract since non-sugar substances oxidized by iodine were found to be present. The method of Kolthoff (1923) was accordingly adopted, and the reducing value obtained after removal of the glucose by oxidation with 0·021 N iodine for 15 minutes at 15° C was accepted as giving the amount of fructose. The difference between this value for fructose and the reducing value of the untreated solution after clearing was taken as the glucose.

In considering whether the values of sucrose, glucose, and fructose, estimated as just described, can be accepted as representing accurately the amounts of these sugars in the extracts, it is necessary to know whether there are in the cleared extracts (*a*) any substances which interfere with the reduction of the copper solution by the true sugars; (*b*) any non-sugar reducing substances.

For the stock used for experiments 2, 3, and 4 of this communication tests in which known quantities of glucose and fructose were added to the extracts showed that the added sugar could be correctly recovered. This is evidence that no substances were present which interfered with the copper reduction.

A valuable test for the presence of non-sugar reducing substances is to determine the reducing value of the extract after the true sugars have been removed by yeast fermentation. Such determinations were not included as a routine part of the sugar estimations, and precise values for the non-sugar reducing material for the various sugar analyses cannot therefore be given. Nevertheless from a few fermentation determinations made on the stock used in experiments 2, 3, and 4, and from subsequent work on other stocks of potatoes we can assess the extent to which the sugar values to be given here are affected by the non-sugar reducing material.

It is not proposed at this stage to give details of the effects of the non-sugar reducing material on the estimation of sugars in potato extracts. It must suffice to state that in the samples of potatoes with which we shall be concerned the amount of non-sugar reducing material was low relative to the concentration of the hexose sugars. Under these conditions we have shown that the estimated values of glucose and sucrose are but little if at all affected by the presence of the non-sugar reducing material. On the other hand the estimated values of fructose include the reducing value of the non-fermentable material and should be corrected for this. The amount of the correction will be stated in considering the experimental results.

## 3—THE PRIMARY DATA OF DRIFT OF RESPIRATION AND OF THE DIFFERENT SUGARS AT VARIOUS TEMPERATURES

In this section it is proposed to set out the data for the drift of respiration and of the different sugars with time, at various temperatures, in the form of respiration/time and sugar/time curves; in the next section the forms of respiration/sugar relations derived from these data will be considered.

It is desirable to state at the outset that the temperatures used to produce sweetening were selected so as to avoid the development of *low temperature depression* as far as possible. A temperature of −1° C, at which the *depression* probably does not develop (*see* Parts 1 and 2), was used for experiment 1, but for certain reasons it was thought preferable to carry out the later experiments at +1° C. Only short periods at +1° C were made use of, in order to avoid the development of *depression.*

*Experiment* 1, 1930. *Drifts of R** *and S at* −1° *C, fig.* 1—To obtain data for the drifts of respiration and of the sugars with time in potatoes changed from 10° C to −1° C a number of samples was selected from a stock which had been kept at 10° C for several months after lifting, precautions being taken in sampling to ensure that each sample was representative of the range in size shown by the stock. Two samples—the respiration samples—were placed in respiration jars, and these and the remaining samples were left undisturbed at 10° C for 12 days before the experiment commenced in order to allow recovery from the disturbing effects of the handling during sampling. All the samples except two were then transferred to −1° C, the two samples being immediately analysed to provide sugar values at the time of the change to −1° C. (For convenience in construction of the graphs, these values are plotted not exactly at zero days in fig. 1 but 1 day to the left of the ordinate axis.)

At −1° C samples were taken for sugar analysis after periods of 1, 2, 4, 6, 10, 14, 20, 26, 34, 45, and 50 days respectively. Each sample provides in fig. 1 values for sucrose (crosses) and hexoses (triangles); and through these individual values, compound curves (interrupted lines) illustrating the drifts of these sugars with time have been built up.

---

* For brevity the symbols R and S are used to express " rate of respiration " and " sugar concentration " respectively.

Beginning at the time of the change to −1° C daily readings were taken of the respiration of the two respiration samples, and the mean R values for these two samples at the various times at which samples were

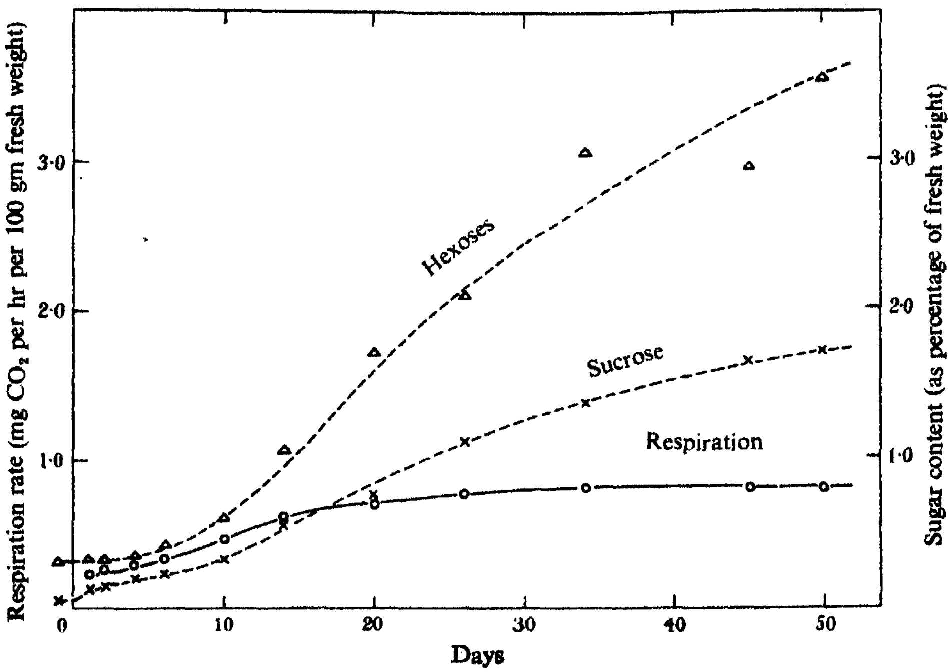


FIG. 1—Experiment 1. Drifts of respiration, ○, sucrose, ×, and hexoses, △, with time in potatoes changed from 10° C to −1° C. Samples were taken for determination of S values after 1, 2, 4, 6, 10, 14, 20, 26, 34, 45, and 50 days at −1° C. S values for each sample are shown by cross (sucrose), and triangle (hexose), at appropriate time. For each time is also plotted mean R value, open circle, of the two respiration samples, whose respiration was determined throughout entire period at −1° C. Points plotted to left of ordinate axis are mean S values of two samples analysed at time remaining samples were changed from 10° C to −1° C and show S at zero time.

taken for sugar analysis, are plotted in fig. 1 (circles). Through these values a compound curve—(continuous line)—has been built up to show the drift of R.

From fig. 1 it is evident that up to about 12 days at −1° C the curves for the respiration, the sucrose, and the hexoses rise very similarly. After 12 days, however, the respiration ceases to rise as rapidly as the sugars, and is approximately level from 30 to 50 days while both sucrose and the hexoses continue to increase.

The consideration of the respiration/sugar relations for this and the following experiments will be postponed to the next section but it is as well to emphasize here that in order to ascertain whether respiration is more closely related to one sugar than to another, data must be obtained in which the relative proportions of the different sugars change during sweetening or de-sweetening. If with changes in the proportions of the sugars the respiration shows a closer correlation with one sugar than with the others, we shall have evidence that this sugar is more directly associated with respiration than the other sugars. Particular interest thus attaches to changes in the relative proportions of the different sugars, and to show the magnitude of these changes, sucrose/hexose quotients, calculated from the respective values of sucrose and hexose given in fig. 1, have been plotted against time at −1° C in fig. 5, A. This figure shows that the quotient increases rapidly during the first 2 days at −1° C, but subsequently remains nearly constant.

In this experiment the change of temperature from 10° C to −1° C results in a rapid change in the sucrose/hexose quotient from the low 10° C value to a higher value; this " transition " is however of short duration and is not associated with any large divergence of the sucrose and hexose values. In the following experiments the transference from one temperature to another results in much more pronounced " transitional " effects on the sucrose/hexose quotient than in the present experiment, and the " transitional " changes of the quotient are associated with considerable divergence in the values of sucrose, and hexose. In these cases then the opportunity is afforded to ascertain whether with divergence of the values of sucrose and hexose, respiration shows changes in definite relation with one sugar or with the other.

In the following experiments the methods of obtaining data for the drifts of R and of S with time, and the presentation of the data in the figures, are essentially similar to those adopted in experiment 1. Restatement of the experimental procedure is thus unnecessary, and the detailed legend given in fig. 1 will not be repeated.

*Experiment* 2, *February*, 1933. *Drifts of R and S with time at* +1° *C, fig.* 2—The data for a different stock of potatoes from that used in experiment 1, and with +1° C as a sweetening temperature instead of −1° C, are shown in fig. 2. The respiration and sucrose again increase nearly proportionally for some days, and then the respiration increase becomes gradually slower, while sucrose continues to increase rapidly. The drift of the hexose sugars (glucose and fructose were here determined separately, and both values are shown in fig. 2) is, however, markedly different from the drift in experiment 1, the concentration falling initially and not increasing until after about 5 days at +1° C.

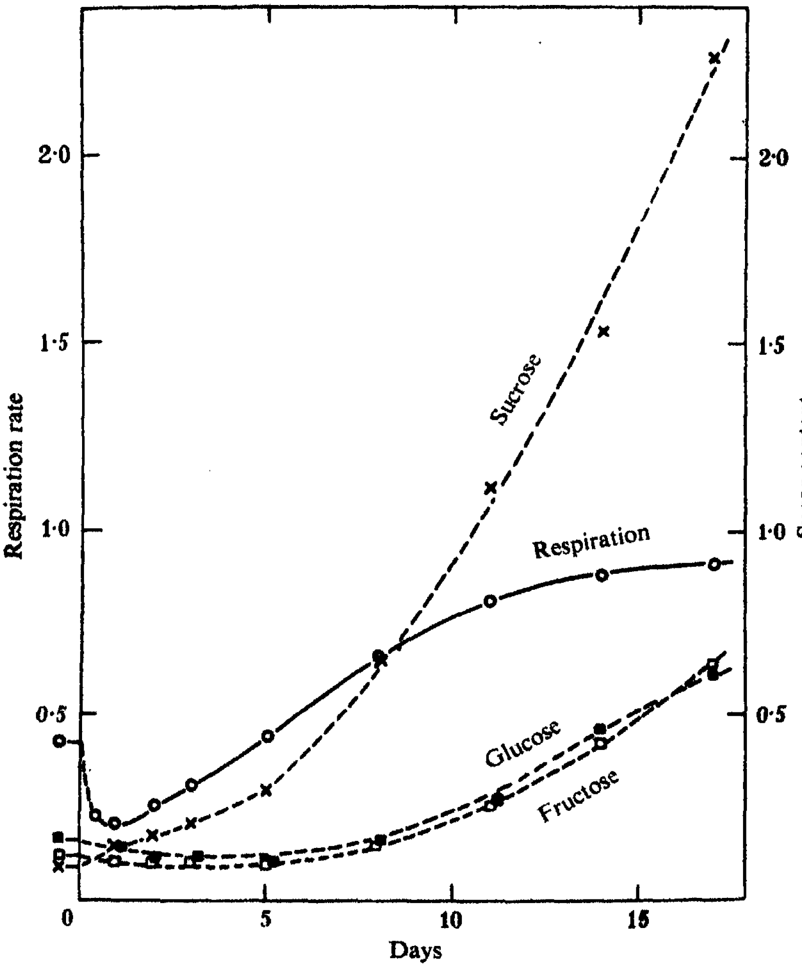


FIG. 2—Experiment 2. Drifts of respiration, sucrose, glucose, and fructose with time, in potatoes changed from 10° C to +1° C. × sucrose; ○ respiration; ■ glucose; □ fructose.

The changes in the sucrose/hexose quotient are shown in fig. 5, A. The quotient increases rapidly for about 10 days and then decreases slowly.

The relative proportions of glucose and fructose change little during sweetening or de-sweetening in the experiments described in this communication and a quotient for these sugars has accordingly not been plotted.

*Experiment* 3, *March,* 1933. *Drifts of R and S with time at* +1° *C, fig.* 3—In order to investigate in greater detail the striking change in the proportions of sucrose and the hexose sugars shown in experiment 2 and to eliminate a possible weakness of the technique in that experiment, a further experiment was made a month later with potatoes from the same stock at 10° C, and again using +1° C as a sweetening temperature. In experiment 2 the drift of the respiration is constructed from daily respiration measurements of the two respiration samples; the respiration of the samples used for sugar analysis was not determined. In the present experiment the respiration of each sample was determined at the time of sugar analysis, and thus the pairs of R and S values at each point in time are strictly comparable.

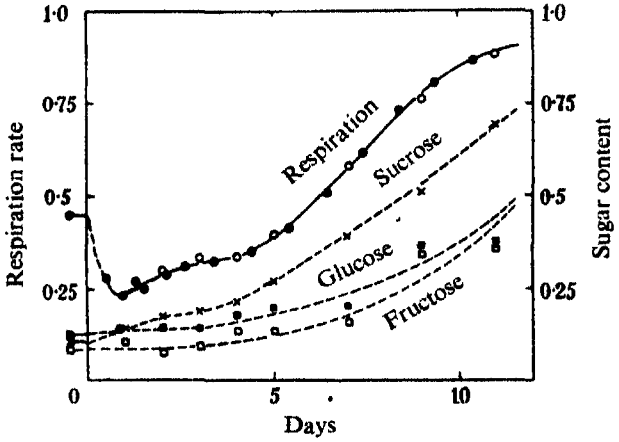


FIG. 3—Experiment 3. Drifts of respiration, sucrose, glucose, and fructose with time in potatoes changed from 10° C to +1° C. ○ = R values of sugar samples at time of analysis; ● = R values of control sample whose respiration was measured at intervals throughout period at +1° C; ■ glucose; □ fructose; × sucrose.

The forms of the drifts of R and the different sugars, fig. 3, differ somewhat from those in experiment 2, fig. 2, but the essential features of that experiment are repeated. Again R and sucrose increase similarly, and it should be noted that the drifts of sucrose and R do not show smooth upward curves; there is a slackening in the rate of increase of sucrose between 2 and 4 days at +1° C, as compared with the previous and subsequent rates; this step in the sucrose curve is correlated in time with a similar step in the drift of R. In contrast with sucrose, glucose, and fructose do not increase until after about 3 days at +1° C, so that there is a rapid increase in the sucrose/hexose quotient, fig. 5, B.

*Experiment* 4, *March*, 1933. *Drifts of R and S with time at* 10° *C, fig.* 4—The special feature of experiments 2 and 3 is that sucrose increases during the first few days at +1° C without a corresponding increase in the hexose sugars. It seemed possible that this increase in the sucrose/hexose quotient might be reversed if potatoes, which had been exposed for several days at +1° C were returned to 10° C. The present experiment tests this point.

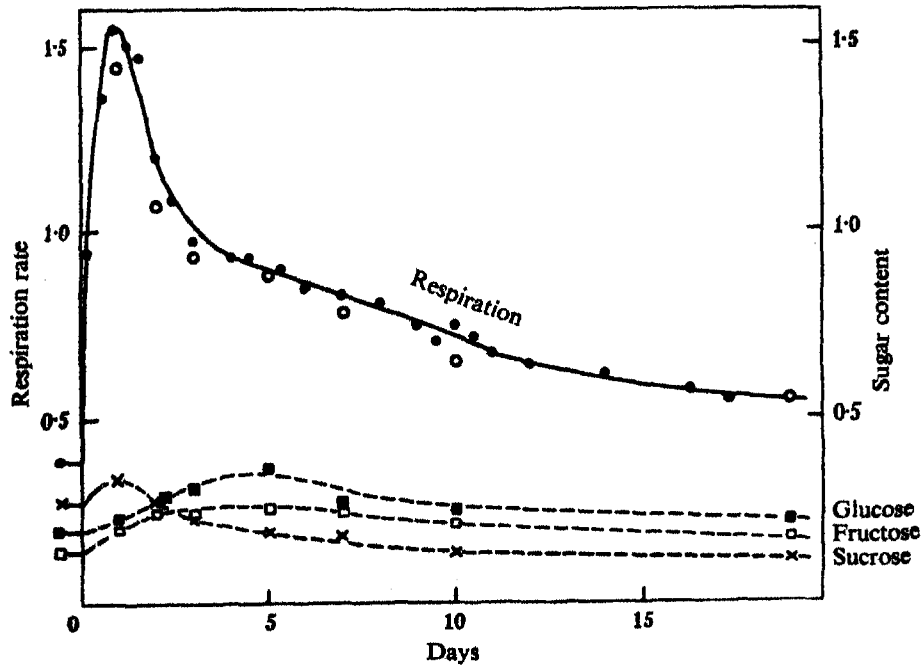


Fig. 4—Experiment 4. Drifts of respiration, sucrose, glucose, and fructose with time in potatoes changed from +1° C to +10° C. ○ = R values of sugar samples at time of analysis; ● = R values of control sample whose respiration was measured at intervals throughout period at 10° C; ■ glucose; □ fructose; × sucrose.

The samples used were exactly comparable with those taken for experiment 3, and were placed in respiration jars at 10° C and transferred to +1° C at the same time as those for experiment 3. The early part of fig. 3 can thus be assumed to represent the drifts in R and S for the samples of experiment 4 during their initial period at +1° C.

After 5 days at +1° C the samples were returned to 10° C, and the subsequent drifts of R and S at 10° C are recorded in fig. 4. The points in fig. 4 to the left of the ordinate axis are the values for the 5th day at +1° C from fig. 3, and as explained above, they may be accepted as showing the R and S at the time of change from +1° C to 10° C.

Fig. 4 shows that the initial changes observed at +1° C in experiments 2 and 3 are reversed on returning the potatoes to 10° C. After 1 day at 10° C both respiration and sucrose decrease together. On the other hand glucose and fructose increase for about 3 days and do not begin to fall until after 5 days.

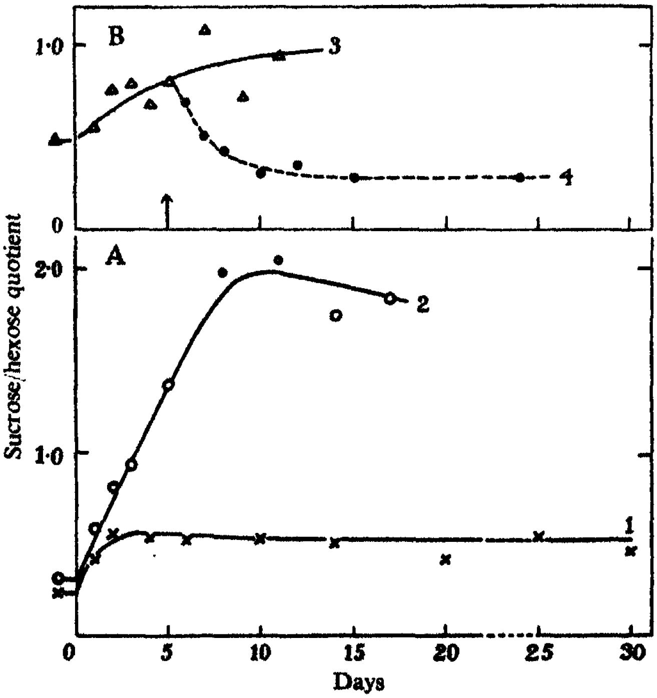


FIG. 5—Drifts of sucrose/hexose quotient with time. A, experiment 1, at −1° C; 2, at +1° C. B, experiment 3, at +1° C; 4, at +10° C.

In fig. 5, B, the sucrose/hexose quotients for the present experiment are plotted so as to continue in time the sucrose/hexose values for experiment 3, *i.e.*, the zero time for experiment 4 is 5 days. The figure shows that the increase of the sucrose/hexose quotient at +1° C in experiment 3 is rapidly reversed on change to 10° C in experiment 4.*

---

* It is desired to draw attention to this evidence of a reversible effect of temperature change on the proportions of sucrose and the hexose sugars. The data suggest that in potatoes there is a balancing mechanism between sucrose and the hexoses which is markedly affected by certain temperature changes.

## 4—THE FORMS OF THE RESPIRATION/SUGAR CURVES CONSTRUCTED FROM THE PRIMARY DATA OF § 3

In this section we propose to survey the data set out in the previous section in order to ascertain which of the sugars, sucrose, glucose, or fructose appears to be most closely related to the actual substrate of respiration. For this enquiry the criterion we shall use is the comparison of the forms of the R/sucrose, R/glucose, and R/fructose curves with the form of the relation between rate of reaction and substrate concentration for an enzyme reaction.

For many enzymatic reactions *in vitro* it is now generally recognized that the rate/substrate relation has the form of a special type of rectangular hyperbola. For such a complex process as respiration *in vivo* we cannot expect precisely this simple relationship for a single enzymatic reaction *in vitro*, but so far as our knowledge goes if we could plot rate of respiration against concentration of respirable substrate, the relation should be an approximation to the enzymatic form. If then our experimental data show a curve of this type when R is plotted against the concentration of a particular sugar we shall have evidence that this sugar is related to the respirable substrate.

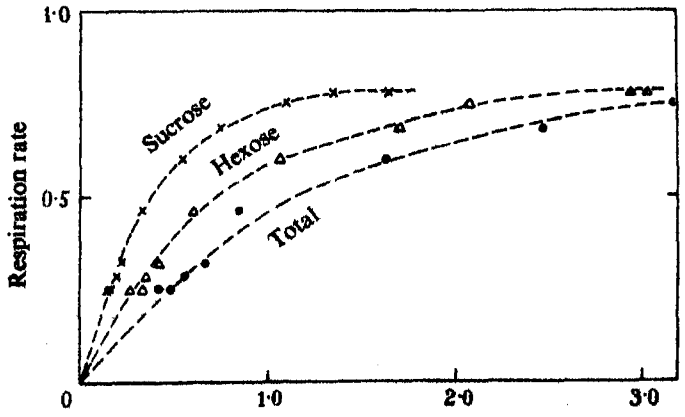


FIG. 6—Experiment 1. Respiration/sugar relations during sweetening at −1° C. × sucrose; Δ hexose; ● total.

*Respiration/sugar curves at* −1° *C. Experiment* 1. *Figs.* 1, 5, 6—The data for experiment 1 are presented as R/sucrose, R/hexose, and R/total sugar curves in fig. 6. The three curves must have the same general form since after the first 2 days at −1° C the relative proportions of sucrose and hexose remain nearly constant during sweetening, fig. 5, A.

The initial rapid increase of sucrose relative to hexose during the first 2 days at −1° C, fig. 5, A, does not materially affect the forms of the respiration/sugar curves though it is large enough to cause the somewhat greater scatter of the first three R/hexose points as compared with the corresponding R/sucrose points, fig. 6, points at R = 0·23–0·28.

The form of each respiration/sugar relation in fig. 6 is broadly that of a rectangular hyperbola, but since the forms are similar for both sucrose and hexoses, we cannot decide from this evidence whether sucrose is more closely related to the respirable substrate, and the observed relation between R and the hexoses is due to an interlinking between sucrose and the hexoses, or vice versa.*

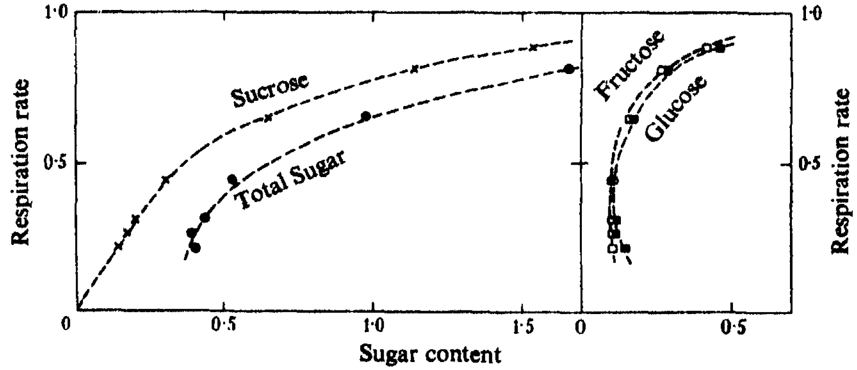


Fig. 7—Experiment 2. Respiration/sugar relations during sweetening at +1° C. × sucrose; ● total sugar; □ fructose; ■ glucose.

*Respiration/sugar curves at* +1° C. *Experiment* 2, *figs.* 2, 5, A, 7, *and experiment* 3, *figs.* 3, 5, B, *and* 8—The results for experiments 2 and 3, for which the same stock was used, are similar and can be considered together. In these experiments the sucrose/hexose quotient, fig. 5, which increased only for the first 2 days in experiment 1, continues to rise for about 10 days. Here then the continued divergence in the proportions of sucrose and the hexoses should enable us to distinguish between the relative influence of these sugars on the respiration.

From figs. 7 and 8 it is evident that the R/sucrose relations are again of the enzymatic type. On the other hand the R/glucose, R/fructose, and R/total sugar relations are of quite a different form.

*Respiration/sugar curves at* 10° *C, experiment* 4, *figs,* 4, 5, B, 9—In experiment 4 there is again a rapid change in the proportions of sucrose and the hexoses, the sucrose/hexose quotient falling quickly, fig. 5, B. The effect on the respiration/sugar relations of this change in the proportion of sucrose to hexose is even more marked than in experiments 2 and 3, figs. 7 and 8, since the amounts of the sugars are initially much higher than for those experiments and the fall of the sucrose/hexose quotient is associated with correspondingly greater changes in the magnitudes of the sucrose and hexoses.

---

* It was evidence of this kind that led us for the time being to adopt total sugar as a measure of the respirable substrate in Parts I and II.

The R/sucrose relation, fig. 9, is nearly linear. The R/glucose and R/fructose relations show no resemblance to the enzymatic curve; indeed these curves are complex, drifting in two different directions since initially glucose and fructose increase as respiration falls, while subsequently all fall contemporaneously.

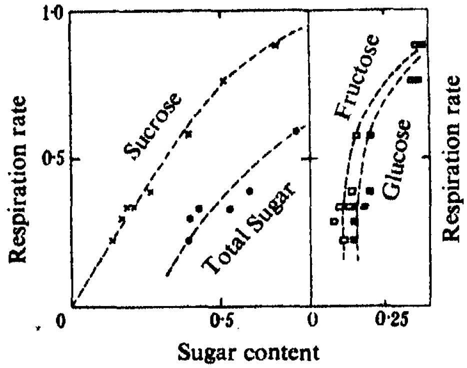


*Fig.* 8—Experiment 3. Respiration/sugar relations during sweetening at +1° C. × sucrose; ● total sugar; □ fructose; ■ glucose.

In fig. 9 in addition to the data for experiment 4, respiration/sugar values have been plotted for four samples, which were initially exactly comparable with those used for experiment 4, but were kept at 10° C throughout, and analysed at intervals during the progress of experiment 4. The respiration/sugar values of these 10° C control samples are shown by the o, Δ, and ● points in fig. 9 (R = 0·45). It is interesting to note that for R/sucrose the curve for the samples, which were partly sweetened at + 1° C, and then transferred to 10° C, open circles, passes through the points for the 10° C control sample, showing that the R/sucrose relation has not been disturbed by the exposure of 5 days at +1° C.* For R/glucose and R/fructose, however, the curves for the samples sweetened at +1° C pass below the 10° C control values.

---

* Had the period at +1° C been somewhat longer the R/sucrose relation at 10° C would have shown *depression* (Parts I and II). The period at +1° C in experiment 4 was intentionally kept short in order to avoid the production of the *depressant*.

*General Survey of Respiration/sugar Data*—We may now consider what general conclusions can be drawn from the four sets of respiration/sugar relations presented in figs. 6–9. The four sets of relations fall into two groups, group A comprising the single set of curves of fig. 6 and group B the curves of figs. 7, 8, and 9. For group A, fig. 6, the sucrose/hexose quotient remains nearly constant during sweetening, and the R/sucrose, R/hexose, and R/total sugar curves accordingly show the same general form, which is broadly that of a rectangular hyperbola. From the evidence of the group A curves the correlation between respiration and sugar is thus equally close for sucrose and for the hexoses.

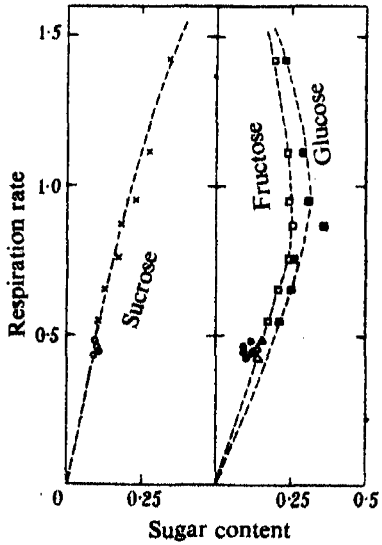


FIG. 9—Experiment 4. Respiration/sugar relations during de-sweetening at 10° C. × sucrose; ■ glucose; □ fructose for samples at 10° C, after 5 days at +1° C; O sucrose; Δ glucose; ● fructose for samples kept at 10° C throughout (*see* text).

On the other hand for the three sets of curves in group B, figs, 7, 8, and 9, the relative proportions of sucrose and the hexoses alter markedly during the changes of sugar content, and the R/sucrose curves have strikingly different forms from the R/glucose, R/fructose, and R/total sugar curves. In each case the R/sucrose curve approaches in form the enzymatic type of group A, fig. 6, *i.e.*, in low sugar the curves appear to be linear through the origin; in higher sugar, fig. 7, the curve tends to flatten parallel to the sugar axis. Moreover the close correlation between R and sucrose is observed both when the total sugar content and the sucrose/hexose quotient are increasing, figs. 7 and 8, and decreasing, fig. 9. In marked contrast the R/glucose and R/fructose curves have no simple standard form and depart markedly from the enzymatic curves. The lack of correlation between respiration and the hexose sugars is best shown in fig. 9 where falling respiration is associated with rising hexoses.

The evidence of the group B curves thus strongly suggests that the respiration is correlated with the concentration of sucrose and that the hexose sugars, glucose, and fructose, are not directly associated with the respiratory process. We cannot be so confident in our interpretation of the group A curves, fig. 6, but in view of the marked lack of correlation between respiration and the hexoses in the group B curves it may be suggested that in group A, fig. 6, the respiration is primarily determined by the sucrose concentration, and that the observed close relation between respiration and the hexoses is due, not to a direct interlinking between hexoses and the respirable substrate, but to a second correlation between sucrose and the hexoses. It would appear that in group B curves this second correlation between sucrose and the hexoses is profoundly disturbed at certain temperature transitions, resulting in marked changes of the sucrose/hexose quotient without affecting the independent correlation between respiration and sucrose.

We cannot consider the relation between sucrose and the hexoses during sweetening and de-sweetening further in this paper. It will suffice to state that the respiration/sugar data given are fully representative of our observations with potatoes. In addition to the experiments described here we have respiration/sugar data for six further sweetening experiments at −1° C or +1° C. In five of these experiments the sucrose/hexose quotient increases markedly during sweetening and the respiration is closely correlated with the sucrose content, *i.e.*, the experiments fall into group B. In the remaining experiment the data are rather scanty but they indicate that the sucrose/hexose quotient remains relatively constant, following a short initial increase, and that the respiration/sugar relations are equally close for sucrose and the hexoses; the experiment thus appears to belong to group A.

In concluding this section we may refer again to the accuracy of the sugar determinations. In § 2 it was stated that fermentation experiments with samples of the stock used for experiments 2, 3, and 4, indicate that the estimated values for fructose include a proportion of non-sugar reducing material. The amount of this material expressed as the equivalent weight of glucose varied in different samples from 0·020 gm to 0·030 gm per 100 gm fresh weight, and the values for fructose should be corrected by subtracting amounts of this order. Since, however, for the reasons already given the extent of the correction cannot be determined precisely it was thought preferable not to correct the values of fructose given in the figures.

In considering to what extent the correction of the fructose values would affect the respiration/sugar relations set out above it is particularly important to note that there is no evidence for any considerable change in the amount of the non-sugar reducing material during sweetening or desweetening. The introduction of a correction for this material could not therefore affect the *direction* of the drifts of fructose, *e.g.*, the initial fall in experiment 2, fig. 2, while sucrose is rising, or the initial rise in experiment 4, fig. 4, while sucrose is falling. There is then so far as we are aware no ground for attributing the lack of correlation between respiration and either glucose or fructose to faulty methods of estimating these sugars.

## 5—THE SIGNIFICANCE OF THE OBSERVED R/SUCROSE RELATION

The evidence presented in the previous sections points strongly to the conclusion that in potatoes sucrose is closely associated with the respirable substrate and that neither the normal glucose nor the normal fructose is immediately concerned in respiration.* We have now to consider what conclusions can be drawn from the forms of the observed R/sucrose relations as to the nature of the respirable substrate, and we propose first to ascertain how closely the R/sucrose relations of experiments 1–4 conform with the curves that express the Michaelis reaction rate/substrate concentration relation for an enzyme reaction *in vitro*.

A special type of rectangular hyperbola is now generally accepted as the form of the rate/substrate relation for many enzymatic reactions *in vitro*, and for the present purpose the equation for the curve may be written:—

$$R = K . E . \frac{S .}{S + Km},$$

where R is the rate of respiration, S the substrate, *i.e.*, sucrose concentration, K a constant, E the enzyme concentration and K*m* the Michaelis constant.

To ascertain how closely the observed R/sucrose relations conform with this equation it is sufficient for the present analysis to convert the equation into the form:—

$$\frac{1}{R} = \frac{1}{K . E} + \frac{Km}{K . E}\left(\frac{1}{S}\right),$$

---

[*] Further evidence of a close relation between the concentration of sucrose and the rate of respiration has been obtained in a study of the respiration/sugar relations of potatoes whose sugar has been increased by treatment with cyanide.

and to plot reciprocals of the observed values of R against the reciprocals of the observed values of S. If the relation between R and sucrose is expressed by a rectangular hyperbola this plotting of 1/R and 1/sucrose should give a straight line.

Reciprocals of the values of R and sucrose for experiments 1–4, have been plotted in fig. 10, and straight lines drawn to show the forms of the 1/R/1/sucrose relations. Except for the low 1/sucrose values of experiments 1 and 2, fig. 10, which show a well-marked departure from linearity the 1/R/1/sucrose relations do not depart far from linearity, the approach being somewhat closer for experiments 3 and 4 than for experiments 1 and 2. It has already been noted in § 3 that because of improved experimental methods the R/sucrose data provided by experiments 3 and 4 are more reliable than those for experiments 1 and 2. We are accordingly justified in attaching more importance to the forms of the 1/R/1/sucrose relations of experiments 3 and 4 than to those of 1 and 2, and in view of the close fit to linearity shown by the former we may draw the general conclusion that except in high sucrose values* the observed R/sucrose

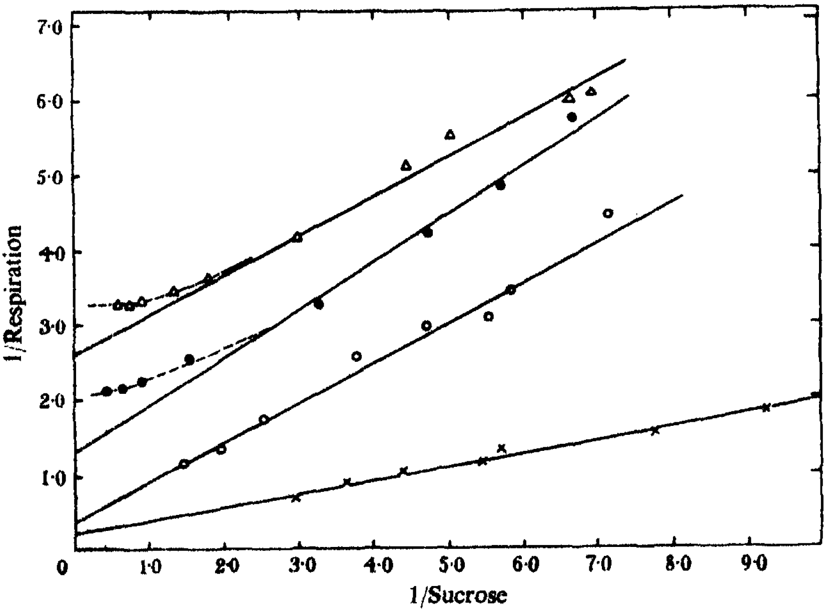
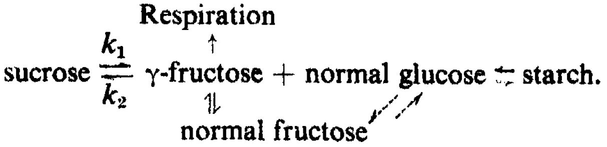


FIG. 10—Relations between 1/respiration and 1/sucrose for experiments 1, 2, 3, and 4. For convenience in graphing all 1/R values of experiments 1 and 2 are increased by addition of 2·0 and 1·0 respectively. Δ exp. 1, at −1° C; ● exp. 2, at +1° C; ○ exp. 3, at +1° C; × exp. 4, at +10° C.

* A depression of the reaction/rate with increasing concentration of substrate is characteristic of many enzymatic reactions *in vitro* and the upward curvature shown

relations conform closely with the form of the enzymatic rectangular hyperbola.

For many plant tissues the respirable substrate is usually considered to be not sucrose, as the R/sucrose relation observed here appears to indicate, but either a hexose sugar or a hexose sugar derivative. This view has been supported recently by the characterization of the fructose in the sucrose molecule as a particularly unstable form of hexose, namely γ-fructose. This led to the suggestion (Onslow, 1931) firstly that γ-fructose derived from sucrose is the respirable sugar and secondly that in certain plant tissues the concentration of γ-fructose, and consequently the rate of respiration, is dependent on the *rate of breakdown* of sucrose. A method of estimating the concentration of γ-fructose in plant extracts has not as yet been developed and these hypotheses are accordingly difficult to test. So far the evidence put forward in favour of the γ-fructose theory (Onslow, 1931; Onslow, Kidd, and West, 1931; Yemm, 1935) can scarcely be considered as decisive, but since the γ-form of fructose is the most reactive form of the two hexose sugars known and for that reason may be more readily broken down in respiration than other sugar forms, we may consider whether the relation between respiration and sugar observed in the potato may be due not to the immediate utilization of sucrose as the respirable substrate, but to a close correlation between the *concentration* of sucrose and the concentration of some product of sucrose-breakdown, *e.g.*, γ-fructose. The following schema indicates a possible system of inter-relationships in which γ-fructose is regarded as the respirable substrate and sucrose as an end-product in the starch-sugar system.



$$\begin{tikzcd}
 & \text{Respiration} & & \\
\text{sucrose} \arrow[r, shift left, "k_1"] & \gamma\text{-fructose} \arrow[l, shift left, "k_2"] \arrow[u] \arrow[d, shift left] & +\ \text{normal glucose} \arrow[r, shift left] \arrow[dl, dashed, shift left] & \text{starch.} \arrow[l, shift left] \\
 & \text{normal fructose} \arrow[u, shift left] \arrow[ur, dashed, shift left] & &
\end{tikzcd}$$

(1)

In considering this system we shall assume that the relation between respiration and γ-fructose is of the enzymatic type:—

$$R = \frac{k \,.\, E\,[\gamma \,.\, F]}{[\gamma \,.\, F] + k_m}.$$

Since we have just shown that except in high sucrose concentrations the observed R/sucrose relations also conform closely with this type of

---

by the 1/R/1/sucrose values of experiments 1 and 2, fig. 10, in high sucrose may thus be due to a depression of the respiration rate, which becomes less as the concentration of sucrose decreases.

rectangular hyperbola, it follows that, with high concentrations excepted, [sucrose] must be proportional to [$\gamma$-fructose] if the above system is to provide the observed enzymatic R/sucrose relation. Moreover since the experimental data, apart from experiment 1, show wide changes with time in the sucrose/hexose quotient and but little alteration in the fructose/glucose quotient, the system should also allow for large changes in the proportion of normal fructose to $\gamma$-fructose without disturbing the proportionality between $\gamma$-fructose and sucrose, and between normal fructose and normal glucose.

Since we do not know the values of any of the reaction constants for the above system little purpose would be served by attempting to formulate an equation relating [sucrose] and [$\gamma$-fructose] for the whole system. We shall confine our attention to the reaction:—

$$\text{sucrose} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \gamma\text{-fructose} + \text{normal glucose}. \qquad (2)$$

If this reaction is in equilibrium then:—

$$[\text{sucrose}] = \frac{k_2\,[\gamma\text{-F}] \times [\text{NG}]}{k_1}$$

and

$$[\gamma\text{-F}] \times \frac{k_2}{k_1} = \frac{[\text{sucrose}]}{[\text{NG}]}\,. \qquad (3)$$

In the experimental R/sugar data there are values for R, sucrose, and normal glucose, and we can thus calculate values for $[\gamma\text{-F}] \times \frac{k_2}{k_1}$ from equation (3), and plot these values against the corresponding values of R. The $\text{R}/[\gamma\text{-F}]\frac{k_2}{k_1}$ relations so obtained depart widely from the enzymatic type, and it does not seem possible to account for the departures on the assumption of a lack of equilibrium in reaction (2) owing to production or loss of $\gamma$-fructose in the reactions:—

$$\text{normal fructose} \rightleftharpoons \gamma\text{-fructose} \rightarrow \text{respiration}.$$

So far then as this analysis can be carried it appears unlikely that the system (1) would conform with the observed R/sucrose relations. We do not propose to consider modifications of system (1). Indeed we must admit that our knowledge of the interrelations of the simple sugars and sugar derivatives in plant tissues is too meagre to permit a decision to be made as to the type of relation to be expected between sucrose and $\gamma$-fructose.

We may conclude that it is impossible to deduce from the observed R/sucrose relations what is the nature of the respirable substrate. The evidence we have presented provides a strong indication that sucrose is either itself the respirable substrate or is closely linked with this substrate. The suggestion that sucrose is itself the respirable substrate seems less unreasonable when it is considered that the γ-fructose in sucrose may form an active wing of the sucrose molecule, and may be the part of the molecule which is oxidized.

So far as we are aware the data here presented constitute the first published evidence of a broadly enzymatic relation between the respiration of a plant tissue and the concentration of a particular internal constituent sugar. It is for further studies to show whether the sugar here found to be most closely related to respiration, namely sucrose, is actually the respirable substrate. It should be emphasized that we have directed attention to the possibility that γ-fructose is the respirable substrate chiefly because of the known reactivity of this hexose form. It may well be that another breakdown product of sucrose is the respirable substrate, and further evidence on the reactivity of other sugar forms and derivatives is needed.

Our investigation suggests that in the potato the respiration/sugar relations can be more readily analysed than in other plant materials. One reason for this appears to be that at least in the early periods of storage, the huge reserves of starch supply all the sugar that is required for respiration and starvation effects do not occur. On the other hand with materials such as cut barley leaves, Yemm (1935), starvation effects predominate, and the attempt to determine the nature of the respiratory substrate appears to be complicated by exceedingly rapid changes in the nature of the supply for respiration.

## CONCLUSION

This paper forms Part III of a communication (Barker, 1933) dealing with the relation between respiration and the concentration of extractable sugar, the latter being varied by exposing the material at different temperatures. In Parts I and II the concentration of total sugars was provisionally adopted as a measure of the respirable substrate; in this Part we take up the question of the relation between respiration and the individual sugars, sucrose, glucose, and fructose.

Evidence is presented which shows that the forms of the respiration/glucose, respiration/fructose, and respiration/total sugar relations vary over a wide range according to the changes of the sucrose/hexose quotient during sweetening and de-sweetening. On the other hand the respiration/sucrose relation both during sweetening and de-sweetening, and also while the sucrose/hexose quotient was either increasing or decreasing, conformed closely with the enzymatic rectangular hyperbola for the rate of reaction/substrate relation for an enzyme reaction *in vitro*.

There is thus strong evidence that sucrose is closely related to the substrate supply for respiration and that neither glucose nor fructose is directly associated with this function.

The bearing of the above observations on our knowledge of the nature of the respirable substrate is discussed.

In samples of low sugar content the sucrose/hexose quotient is shown to be markedly affected by changes of temperature.

I am deeply indebted to Dr. Blackman for his stimulating interest in this investigation. I wish to thank Mr. G. E. Briggs for his assistance in the analysis of the data.

I wish also to acknowledge the invaluable help of Mr. H. A. F. Jackson who conducted the sugar analyses and of Mr. A. E. Porter who carried out the respiration measurements and other experimental work.

## REFERENCES

Barker, J. (1933). ' Proc. Roy. Soc.,' B, vol. **112**, p. 316.
Hanes, C. S., and Barker, J. (1931). ' Proc. Roy. Soc.,' B, vol. **108**, p. 95.
Kolthoff, J. M. (1923). ' Z. Untersuch. Nahr-u Genussm.,' vol. **45**, p. 141.
Onslow, M. W. (1931). " Principles of Plant Biochemistry," Part I.
Onslow, M. W., Kidd, F., and West, C. (1931). ' Food Investigation Board, Ann. Rep.,' 1931, p. 73.
Yemm, E. W. (1935). ' Proc. Roy. Soc.,' B, vol. **117**, p. 504.

581 . 144 . 2 : 576 . 809 . 538

# The Action of Sodium Nitrate upon the Infection of Lucerne Root-Hairs by Nodule Bacteria

By H. G. THORNTON, Bacteriology Department, Rothamsted Experimental Station

(*Communicated by Sir John Russell, F.R.S.—Received* October 23, 1935)

[PLATES 11, 12]

## 1—INTRODUCTION

The fact that nitrates and ammonium salts harmfully affect nodule formation and the subsequent nitrogen fixation in legumes is well known, and the problem has formed the subject of an extensive literature, references to which are quoted by Giöbel (1926), by Fred, Baldwin, and McCoy (1932), and by Allison and Ludwig (1934) and need not be given here *in extenso*. The technical difficulties involved in analysing the action of nitrate upon the association of host and nodule organism are considerable; moreover, the great variation in cultural methods used has made it difficult to compare the results of different workers and has sometimes produced apparent discordance between them. The following facts appear, however, to have been established:

1. Nitrates in sufficient concentration reduce the number of nodules formed and may even entirely inhibit their appearance. Doses of from 5 to 175 mg of nitrate nitrogen per litre have been found entirely to stop nodule formation in agar cultures of legumes (Hiltner, 1900; Flamand, 1903–4; Marchal, 1901; Hills, 1918). In sand and soil cultures the inhibiting doses have been found to range from 21 to 488 mg of nitrogen per litre of soil moisture or of solution in the sand (Fred and Graul, 1916; J. K. Wilson, 1917; Hills, 1918; Strowd, 1920; Ohkawara, 1928). The highest of these figures in both agar and pot cultures are probably excessive, since they refer to initial doses, whereas nodule formation may sometimes have occurred when a weaker concentration, initially inhibitive, was further reduced by assimilation.
2. With doses of nitrate that reduce nodule numbers but do not entirely stop nodule formation, this reduction in nodule numbers is not correlated with the reduction in root-mass, if any. There is an actual reduction in the number of nodules per unit mass of root as compared with the control plants without nitrate (Thornton and Nicol, 1935).

3. The nodule bacteria are not directly injured by nitrates at concentrations sufficient to stop nodule formation; thus Müller and Stapp (1925) found that 0·2% of potassium, sodium, and ammonium nitrates in the media, were actually stimulating to nodule bacteria in culture.

4. In addition to their influence in reducing nodule numbers, nitrates reduce the growth of such nodules as are formed. At moderate concentrations this effect of nitrates upon nodule size is more marked than is their influence on nodule numbers (Giöbel, 1926; Thornton and Nicol, 1935).

5. Nitrates reduce the quantity of nitrogen fixed not only per plant but also per nodule (Giöbel, 1926).

These facts enable us to eliminate certain classes of hypotheses that have been put forward to explain the injurious effect of nitrates upon nodule formation and activity. Thus the cause of this injurious effect, if a single one, cannot be outside the plant, since it affects nodule growth as well as root infection. It cannot, on the other hand, be concerned wholly with the activity of the bacteria within the nodule, since the appearance of nodules may be entirely prevented. The relatively high tolerance of the nodule bacteria themselves towards nitrate makes any theory of its direct toxic action on the bacteria most improbable. It is difficult to escape the conclusion, therefore, that nitrates change the growth and physiology of the host plant in some way that is detrimental to infection by the nodule organism and to subsequent nodule growth and activity. There is some evidence that this change is connected with a reduction in the amount of available carbohydrate occasioned by the increased nitrogen supply, or perhaps rather with a narrowing of the ratio of available carbohydrate to nitrogen within the tissues of the plant. Thus Strowd (1920) found that where nitrate was present in the plant juice, the sugar content was markedly reduced. Orcutt and Wilson (1934) studied the effects of different doses of nitrate upon the content of reducing sugars and sucrose in the sap of soybeans. They found that effects were by no means simple, but in general they concluded that doses of nitrate strong enough to inhibit or reduce nodule formation also decreased the sugar content of the sap.

Fred and Wilson (1934) have shown that the harmful effects of nitrate upon nodule formation could be mitigated by increasing the $CO_2$ content of the atmosphere surrounding clover plants. Allison and Ludwig (1934) obtained a similar mitigation in nitrate effect by adding sucrose to cultures of lucerne grown in sand supplied with a food solution containing nitrate.

This suggestion that the action of nitrate upon nodule formation is an indirect one dependent on a reduction in the carbohydrate available to the roots still leaves unexplained the actual mechanism of the action. Allison and Ludwig sought to find this mechanism in the reduction of root growth. The general reduction in root growth is, however, insufficient to account for the full effect of nitrate upon nodule formation, since the nitrate greatly reduces the number and the volume of nodules per unit mass of root (Thornton and Nicol, 1935). The nitrate must therefore produce some special changes more particularly inhibitive to nodule formation and activity than is the mere reduction in size of the root system.

It seemed that the mechanism of the nitrate effect could only be discovered by a detailed comparison of the processes of root infection by the nodule organism and the subsequent nodule formation in plants supplied with nitrate and in control nitrate-free plants. Since nitrates affect both the numbers of nodules formed and also their size and activity the investigation fell naturally into two divisions: a study of the influence of nitrate upon (1) the process of root infection by the bacteria, and (2) the growth and structure of the nodule. This natural sub-division of the work does not, of course, prejudice the question as to whether the mechanism of the nitrate effect is similar or different at the two stages. The investigation described here deals with the action of nitrate upon the process of root infection.

## 2—THE NORMAL COURSE OF ROOT INFECTION

The normal avenues of infection by the nodule organism are the root-hairs, and the general course of the process is well known. The earliest stage that can be detected is the formation of a small colony of the bacteria close to the distal end of the root-hair, fig. 6, Plate 11. The presence of this colony causes irregular growth of the root-hair, the tip of which usually becomes curled over into a short spiral. The bacterial infection takes place in this deformed region and manifests itself by the formation of a thread of bacterial zoogloea passing down the interior of the root-hair, fig. 7, Plate 11. The exact method of entry is still not clear, but it seems that the deformed growth of the root-hair in some way weakens the cell wall so as to enable the bacteria to force an entry.

Only a fraction of the curled root-hairs actually becomes infected but the evidence of numerous observations shows that root-hairs are not infected unless deformed (McCoy, 1932). Deformation of the root-hairs is a necessary prelude to infection. Where nodule bacteria are present on and amongst the root-hairs, other types of bent and irregular root-hair growth are also far more frequent than on plants growing aseptically (McCoy, 1932). Root-hair deformation is caused by a secretion of the bacteria which can be obtained in sterile solution by the filtration of a suspension of nodule bacteria.

Such a filtrate is capable of deforming the root-hairs of the host plant. This fact was recorded by Hiltner (1900) and has been confirmed by McCoy (1932) and by numerous observations by the present author.

In studying the inhibitive action of nitrate upon root infection by the nodule organism, it was first necessary to determine whether it influenced the deformation of the root-hairs or their subsequent infection by the bacteria.

## 3—TECHNIQUE

The observations recorded here were all made on the roots of lucerne seedlings grown in sterilized agar medium in wide (3 cm diameter) test tubes. The following basal medium was used throughout the experiments and is referred to below as "Seedling agar":—

| | |
|---|---|
| $K_2HPO_4$ | 0·5 gm |
| $MgSO_4 . 7H_2O$ | 0·2 ,, |
| NaCl | 0·1 ,, |
| $Ca_3 (PO_4)_2$ | 2·0 ,, |
| $FeCl_3$ | 0·01 ,, |
| Agar | 10·0 ,, |
| Water | 1 litre |

To this medium the compounds to be tested, such as nitrate, were added in the concentrations given for each experiment. Where the actions of living bacteria were to be studied, 30 ml of the medium were placed in each tube and the tubes plugged with cotton wool and sterilized in the autoclave. The melted medium was then brought to 42° C in a thermostat bath and each tube was inoculated with a loopful of a culture of the lucerne nodule organism grown upon slopes of root extract agar (*see below*).

In the experiments in which sterile bacterial secretions were used, these were obtained by the following method. Cultures of nodule bacteria were grown in wide test tubes on slopes of lucerne root extract agar, having the following composition:—

| | |
|---|---|
| $K_2HPO_4$ | 1·0 gm |
| $MgSO_4 . 7H_2O$ | 0·2 ,, |
| $CaCl_2$ | 0·1 ,, |
| NaCl | 0·1 ,, |
| $FeCl_3$ | 0·02 ,, |
| Agar | 15·0 ,, |

Aqueous extract of lucerne roots, calculated to contain 2·0 gm of dry matter,

| | |
|---|---|
| Water | To 1 litre |
| Sucrose | 10 gm |
| $CaCO_3$ | 5 ,, |

The last two ingredients were added after melting the agar. After about 4 weeks' incubation at 25° C the bacterial growth was washed off into a flask, using for this purpose about 15 ml of distilled water per tube. The suspension thus produced was shaken for 5 minutes by hand and was then cleared by passing it through a pad of filter paper about 2 inches thick set up as shown in fig. 1. In preparing this pad, wet filter paper, torn into fragments of about 1 sq cm, was placed in the wide tube and compressed by means of a glass ramrod, the surplus water being removed by applying suction with the filter pump. The bacterial suspension was then passed through the pad under reduced pressure. After this preliminary clearing the suspension was passed through an L1 Chamberland filter-candle and finally sterilized by passage through an L5 filter candle into a previously sterilized flask. Platings have shown that the filtrates thus obtained were sterile. It was sometimes found necessary to dilute the bacterial suspension somewhat, when it was especially viscous. In consequence the final strength of the secretions varied to some extent as between different experiments. The same batch of filtrates was, however, used throughout each individual experiment, save in the one experiment described in § 5, p. 485, where two different filtrates were compared. In that experiment special care was taken to make the two suspensions of similar density. The seedling agar, to which filtrates containing the bacterial secretions were added, was made up at 1½ times the normal strength and 20 ml of this medium were placed in each tube. After sterilizing, the tubes were brought to 42° C and 10 ml of the filtrate were added aseptically to the melted agar in each tube.

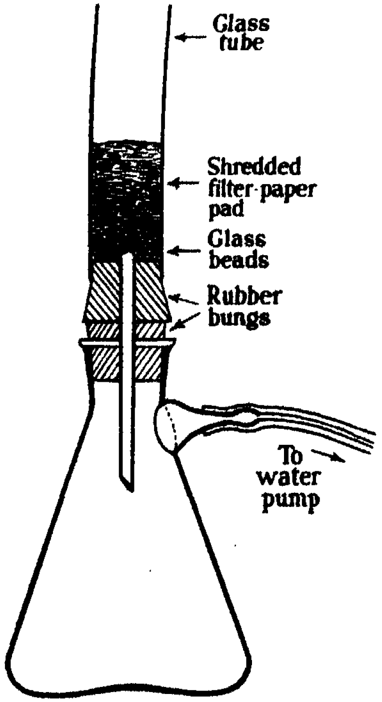


Fig. 1—(Scale 1 : 4).

After cooling the medium, the seed was sown as follows. Lucerne seeds were sterilized externally by immersion for 3 minutes in absolute alcohol and for 3 minutes in 0·2% aqueous $HgCl_2$ solution and washed in four changes of sterile water. Three or four of the seeds were introduced aseptically on to the agar surface and arranged round the inner surface of the glass, so that the roots might tend to grow between the agar and the glass. The tubes were incubated for 2 days at 25° C to hasten germination and were thereafter kept in a glasshouse, the lower half of each tube being encased in a sheath of dark paper to keep light from the roots.

The root-hairs were examined when root growth appeared adequate, usually after about 4 weeks. The examination was carried out as follows:—

The plug of agar was shaken out of the tube and 6 to 8 lengths of root about 2 cm long were carefully lifted from the surface of the agar that had been in contact with the side of the tube. These lengths of root were immersed for 2 minutes in weak aqueous methylene blue, washed and mounted in water and examined under a Leitz No. 3 objective and × 10 eyepiece. Counts of the number of straight and deformed root-hairs were made from microscope fields taken at random, save that the terminal half-centimetre of each root, bearing only very young root-hairs, was not examined.

From the figures thus obtained, the percentages of deformed root-hairs were calculated. The individual tube was taken as the unit for the calculation of standard errors. The number of replicate tubes varied in different experiments but is given below in the various tables.

## 4—THE ACTION OF LIVING NODULE BACTERIA ON ROOT-HAIRS IN THE PRESENCE AND ABSENCE OF NITRATE AND AMMONIUM SALTS

In the first experiment, agar tube cultures of lucerne were set up with the following percentages of sodium nitrate, each in triplicate:—0·125, 0·25, 0·5, 0·75, and 1·0. Six control cultures without nitrate were also included. All the tubes were inoculated with an active strain of lucerne nodule bacteria. The tubes were sown on June 24, 1932. After 3 weeks' growth, an examination was made of two control cultures and one culture from each of the sets receiving the four weakest doses of nitrate. After 2 months, the remaining four control tubes, and two tubes of the sets receiving 0·25, 0·75, and 1·0% nitrate, were examined. The results are set forth in Table I. Amongst the sets receiving nitrate, the percentage of deformed root-hairs showed no correlation with the dose of nitrate

## TABLE I—ACTION OF LIVING NODULE BACTERIA IN DEFORMING ROOT-HAIRS OF LUCERNE GROWN IN AGAR WITH AND WITHOUT $NaNO_3$

| | Series and treatment | Tube No. | Total number of root-hairs examined | Number of infected root-hairs | % of root-hairs | | |
|---|---|---|---|---|---|---|---|
| | | | | | Straight | Bent | Curled* |
| Counts after 3 weeks' growth. | No nitrate | I | 289 | 3 | 40·83 | 32·18 | 26·99 |
| | „ | II | 486 | 5 | 37·65 | 45·06 | 17·28 |
| | | Mean | | 4<br>=1·03% | 39·24<br>±1·59 | 38·62<br>±6·44 | 22·14<br>±4·85 |
| | + nitrate | | | | | | |
| | 0·125% | I | 211 | 0 | 91·0 | 9·0 | 0 |
| | 0·25% | I | 192 | 0 | 96·88 | 2·6 | 0·52 |
| | 0·5% | I | 167 | 0 | 87·43 | 11·38 | 1·19 |
| | 0·75% | I | 264 | 0 | 97·35 | 2·65 | 0 |
| | | Mean | | | 93·165<br>±2·40 | 6·408<br>±2·24 | 0·427<br>±0·89 |
| Counts after 2 months' growth. | No nitrate | III | 856 | 8 | 54·67 | 36·80 | 8·53 |
| | | IV | 639 | 6 | 46·79 | 45·07 | 8·14 |
| | | V | 559 | 5 | 46·15 | 46·87 | 6·98 |
| | | VI | 599 | 7 | 56·26 | 39·73 | 4·01 |
| | | Mean | | 6·5<br>=0·98% | 50·968<br>±2·62 | 42·117<br>±2·33 | 6·915<br>±1·023 |
| | + nitrate | | | | | | |
| | 0·25% | II | 347 | 0 | 96·25 | 3·75 | 0 |
| | | III | 225 | 0 | 90·67 | 7·56 | 1·77 |
| | 0·75% | II | 220 | 0 | 95·00 | 5·00 | 0 |
| | | III | 113 | 0 | 92·04 | 7·08 | 0·88 |
| | 1·0% | II | 280 | 0 | 97·86 | 2·14 | 0 |
| | | III | 250 | 0 | 98·8 | 1·20 | 0 |
| | | Mean | | | 95·103<br>±1·314 | 4·455<br>±1·05 | 0·442<br>±0·302 |

* In Tables I and IV the term "curled" is applied to root-hairs that show typical curling of the distal extremity, otherwise deformed hairs are referred to as "bent". In other tables the term "deformed" covers both curled and bent root-hairs.

so that it was thought permissible to regard all the tubes receiving nitrate as replicates for the purpose of calculating means and standard errors. The results from individual tubes are, however, given in Table I.

About 1% of the root-hairs examined in the control tubes, both after 3 weeks' and 2 months' growth, were found to be infected, but no infected root-hair was seen in any of the cultures containing nitrate, where over two thousand root-hairs were examined. There was thus an apparently complete inhibition of root-hair infection in the presence of the nitrate.

After 3 weeks' growth, the nitrate reduced the mean percentage of typically curled root-hairs from 22·14 in the control to 0·43, and that of otherwise deformed hairs from 38·62 to 6·41. After 2 months' growth the control tubes showed only 6·92% of curled hairs, but the nitrate reduced this percentage to 0·44 and that of otherwise deformed hairs from 42·12 to 4·46.

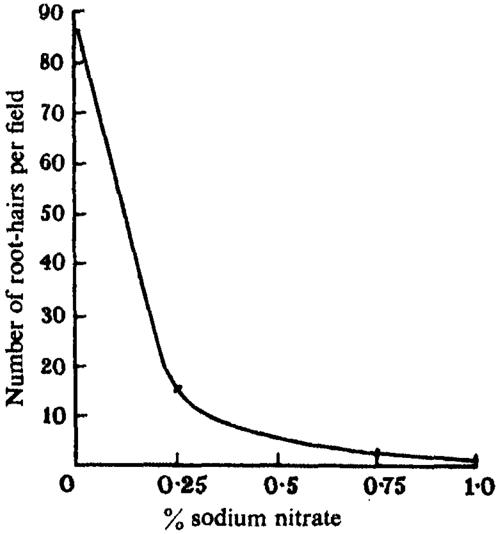


Fig. 2—Mean number of root-hairs per microscope field on lucerne roots grown in different concentrations of nitrate.

The nitrate also greatly reduced the absolute number of root-hairs developing per unit length of root. After 2 months' growth, three segments of root were taken from each of two control tubes and from two tubes from each of the sets receiving 0·25, 0·75, and 1·0% of nitrate, and from each root segment the number of root-hairs in 10 random microscope fields was counted. The mean counts obtained are graphed in fig. 2. The mean number of root-hairs per microscope field was reduced from 86·2 in the control tubes to 15·6 by 0·25% nitrate. This concentration of nitrate thus reduced the number of root-hairs more than 5·5 times as compared with the control. Of these root-hairs the percentage that was typically curled was reduced 15·6 times by the nitrate. Since only curled hairs are normally infected, the chances of infection were thus reduced 85·8 times by a dose of 0·25% nitrate. Of the root-hairs that are curled only a small number actually becomes infected and leads to the production of nodules (McCoy, 1932). In the present experiment the cultures supplied with nitrate showed no nodules but the control cultures after 2 months' growth bore only from 8 to 13 nodules per tube. An 85-fold reduction in the number of curled root-hairs is thus amply sufficient to account for the complete absence of nodules in the sets supplied with nitrate.

The action of nitrate in checking the deformation of lucerne root-hairs by living nodule bacteria was confirmed by a second experiment using seedling agar with 0·15% sodium nitrate and including control sets with bacteria but no nitrate and with neither nitrate nor bacteria. This experiment was commenced on April 21, 1933. The percentages of deformed, *i.e.*, curled and otherwise bent, root-hairs found after about 1 month's growth, are shown in Table II. Here the nitrate reduced the percentage of root hairs deformed in the presence of the bacteria to a figure not significantly higher than that of the sterile set. Further confirmation of the depressing action of nitrate on root-hair deformation by living bacteria is provided by the experiment described in § 5.

TABLE II—ACTION OF SODIUM NITRATE IN REDUCING ROOT-HAIR DEFORMATION BY LIVING BACTERIA. LUCERNE GROWN IN AGAR

| Treatment | Number of replicate tubes | Total number of root-hairs examined | % of root-hairs deformed |
|---|---|---|---|
| No nitrate; uninoculated controls .. | 6 | 1610 | 13·2 ± 1·16 |
| No nitrate + lucerne nodule bacteria | 6 | 2043 | 63·78 ± 3·21 |
| + 0·15% sodium nitrate + lucerne nodule bacteria ................ | 3 | 1122 | 16·53 ± 2·46 |

The action of an ammonium salt upon root-hair deformation by living nodule bacteria was also tested in an experiment in which 0·1% of ammonium sulphate was added to seedling agar and the tubes sown with lucerne and inoculated with an active strain of the nodule organism. Control sets with and without bacteria, both without ammonium sulphate, were included. The lucerne was sown on September 5, 1933; examination after 2 months' growth gave the percentages of deformed root-hairs shown in Table III. Counts of root-hairs from random microscope fields were also made and gave the results shown in this table. The ammonium sulphate greatly reduced the deformation of the root-hairs by the bacteria and also reduced the number of root-hairs produced to the level of the uninoculated control. Ammonium sulphate thus appears to be similar to nitrate in its action in checking the deformation of root-hairs and in reducing their number, though its action in these respects has not been further investigated up to the present. It would seem, however, that its action in checking the appearance of nodules is due to the same cause that operates with nitrate.

TABLE III—ACTION OF LIVING NODULE BACTERIA ON LUCERNE ROOT-HAIRS IN THE PRESENCE AND ABSENCE OF AMMONIUM SULPHATE

| Treatment | Number of replicate tubes | Estimate of root-hair deformation | | Counts of root-hairs in random microscope fields | |
|---|---|---|---|---|---|
| | | Total number of root-hairs examined | % of root-hairs deformed | Total number of fields examined | Mean number of root-hairs per field |
| Sterile control; no ammonium sulphate ................ | 5 | 3220 | 12·6 ± 3·79 | 177 | 18·9 ± 2·41 |
| + living bacteria; no ammonium sulphate ............ | 5 | 1877 | 69·0 ± 1·93 | 65 | 28·8 ± 2·69 |
| + living bacteria + 0·1% ammonium sulphate ...... | 5 | 1505 | 20·6 ± 2·46 | 87 | 18·1 ± 2·32 |

In the first experiment here described the cultures receiving 0·125% of sodium nitrate showed plentiful nodule bacteria amongst the root-hairs although fewer could be seen in the sets receiving 0·75 and 1·0% nitrate. This confirms the conclusion of Müller and Stapp (1925) that these organisms are able to tolerate concentrations of nitrate which inhibit nodule formation. Since these doses of nitrate do not stop bacterial growth, their action in checking root hair deformation must be due to one of two causes. The nitrate must either (*a*) check the secretion by the bacteria of the substance active in deforming the root-hairs, or (*b*) it must tend to inhibit the action of this substance upon the root-hairs. It was not possible to distinguish between these alternative possibilities in the experiments above described, in which the plant roots were supplied with living bacteria, since both the plant and the bacteria were subjected to the influence of the nitrate. The first possibility could, however, be tested by separating their secretions from bacteria grown with and without nitrate, and applying these sterile secretions to plants grown aseptically in a nitrate-free medium, while the second could be tested by applying the sterile secretions from bacteria grown without nitrate to plants growing with and without nitrate.

### 5—THE EFFECT OF THE SECRETIONS OF NODULE BACTERIA GROWN WITH AND WITHOUT NITRATE

The following experiment was performed to determine whether the presence of nitrate checked the secretion by the bacteria of the substance active in deforming the root-hairs. Two sets of slope cultures of lucerne nodule bacteria were grown in wide test tubes. In one set (*a*) the medium used was lucerne root extract agar without nitrate, while in the other set (*b*) 0·2% of sodium nitrate was added to the same medium. From these two sets of cultures, after 2 weeks' growth at 25° C, bacterial suspensions of similar density were prepared, and sterile filtrates containing the bacterial secretions were obtained as described above. Lucerne seedlings were then grown in tubes containing seedling agar with the following treatments:—1, no addition; 2, inoculated with living nodule bacteria; 3, with the addition of filtrate *a*, and 4, with the addition of filtrate *b*. The tubes were sown with lucerne on August 19, 1932. After 4 weeks' growth, the counts of straight, curled, and otherwise deformed root-hairs yielded the percentages shown in Table IV.

TABLE IV—THE EFFECT UPON LUCERNE ROOT-HAIRS OF THE STERILE SECRETIONS FROM NODULE BACTERIA GROWN WITH AND WITHOUT SODIUM NITRATE

| Treatment | Number of replicate tubes | Number of root-hairs examined | % of root-hairs | | | |
|---|---|---|---|---|---|---|
| | | | Straight | Bent | Curled | Bent or curled |
| Control (no bacteria or secretions) .... | 4 | 2226 | 97·88 | 2·12 ± 0·337 | 0 | 2·12 ± 0·337 |
| Living bacteria grown without nitrate .. | 4 | 1498 | 23·84 | 60·22 ± 4·535 | 15·94 ± 3·47 | 76·16 ± 4·30 |
| + secretions of bacteria grown without nitrate (*a*) .... | 6 | 3172 | 15·10 | 80·93 ± 3·92 | 3·97 ± 0·899 | 84·90 ± 3·15 |
| + secretions of bacteria grown with 0·2% nitrate (*b*) .. | 6 | 3257 | 15·28 | 81·17 ± 3·70 | 3·55 ± 0·799 | 84·72 ± 3·397 |

The living bacteria, owing to their local action near the tips of the root-hairs, produced more typically curled hairs than did the secretions, whose characteristic action is to produce a generally irregular growth of the root-hairs throughout their length (compare fig. 9, Plate 11, with fig. 11, Plate 12). The previous growth of the bacteria on a medium containing 0·2% of sodium nitrate was quite without effect upon the activity of their secretions.

## 5—THE EFFECT OF NITRATE UPON THE ACTION OF THE BACTERIAL SECRETIONS

Since nitrate does not inhibit the production of the active substance by the bacteria, it must check its action upon the root-hairs. To test this, cultures of lucerne nodule bacteria were grown on lucerne root extract agar and their secretions separated from them by filtration. The sterile filtrate containing these secretions was added to tubes of seedling agar in one set (1) without nitrate, and in a second set (2) containing 0·1% of sodium nitrate. Two similar sets (3) and (4) with and without nitrate were inoculated with living bacteria in order to compare the action of these with their sterile secretions. All the tubes were sown with lucerne on August 30, 1932. Counts of straight and deformed hairs after 3 weeks' growth yielded the percentages shown in Table V. In the presence of the sterile bacterial secretions, plants grown without nitrate showed 71·83% of the root-hairs deformed. The addition of 0·1% sodium nitrate to the medium in which the plants were grown reduced this percentage of deformed hairs to 12·78%. With living bacteria, the results were not significantly different. (*See* fig. 9, Plate 11, and figs. 10, 11, and 12, Plate 12.) Nitrate thus produced a similar effect in checking root-hair deformation, no matter whether living bacteria or their sterile secretions were the active agents. The nitrate must therefore act by pre-

TABLE V—ACTION OF THE LIVING NODULE BACTERIA AND OF THEIR STERILE SECRETIONS UPON ROOT-HAIRS OF LUCERNE GROWN WITH AND WITHOUT SODIUM NITRATE

| Treatment | Number of replicate tubes | Total number of root-hairs examined | % deformed root-hairs |
|---|---|---|---|
| With bacterial secretions— | | | |
| (1)—Plants in agar without nitrate | 4 | 1548 | 71·825 ± 4·02 |
| (2)—Plants in agar with 0·1% $NaNO_3$ | 4 | 1801 | 12·78 ± 3·37 |
| With living bacteria— | | | |
| (3)—Plants in agar without nitrate | 4 | 712 | 80·36 ± 3·30 |
| (4)—Plants in agar with 0·1% $NaNO_3$ | 4 | 903 | 14·12 ± 1·79 |

venting the bacterial secretions from affecting the root-hairs. Confirmation of this result was obtained in the two separate experiments described in the next section.

## 6—The Influence of Dextrose in Mitigating the Effect of Nitrate

Recent American work tends to connect the effect of nitrate in arresting nodule formation with its action in reducing the concentration of soluble carbohydrates in the plant. Allison and Ludwig (1934) indeed found that the depressing effect on nodule formation produced by nitrate was lessened when sucrose was present as well as nitrate in the medium surrounding the roots of inoculated lucerne. This raised the question whether the action of nitrate in protecting the root-hairs against deformation by the bacterial secretions could also be lessened by adding a soluble carbohydrate to the medium in which the roots were grown. Where living bacteria are present in such experiments, the interpretation of the results is complicated, since it is likely that these organisms will multiply greatly in the presence of the carbohydrate, so that the increased development of nodules may be due either to the larger number of bacteria around the roots, or to the reduction in the nitrate concentration outside the plant, through bacterial assimilation. These difficulties do not, however, arise where the sterile secretions of nodule bacteria are supplied to plant roots grown aseptically in agar containing nitrate and carbohydrate. To test the effect of supplying both nitrate and carbohydrate to the roots, an experiment was made in which lucerne was grown in seedling agar supplied with a sterile filtrate containing the secretions of nodule bacteria. In one set the medium contained 0·1% sodium nitrate, in a second, 0·1% nitrate and 0·5% dextrose, and in a third set no nitrate or dextrose. A fourth set without bacterial secretions, nitrate, or dextrose was also included. The seeds were sown on September 5, 1933, and the roots examined after 2 months' growth. The percentages of deformed root-hairs that were found are shown in Table VI. The nitrate produced an unusually small effect in reducing the root-hair deformation in this as compared with other experiments, though even in this instance the reduction from 83·1% in the set without nitrate to 42·5% in that receiving nitrate is quite considerable and is very significant. In the presence of nitrate and dextrose the bacterial secretions produced 61·3% of deformed root-hairs. This figure is just significantly higher than the 42·5% found with nitrate without dextrose. The dextrose thus mitigated the depressing action of nitrate on the root-hair deformation.

It was felt, however, that this result needed confirmation owing to the rather small effect of nitrate even without dextrose and to the high standard error of the set supplied with dextrose. Another experiment was therefore commenced on April 21, 1935, with the same treatments. The results after 10 weeks' growth are shown in Table VII. Here the addition of nitrate without dextrose reduced the percentage of deformed root-hairs to 11·2 where that in the set with secretions but no nitrate was 48·97 while the further addition of dextrose restored the percentage to a figure not significantly different from the latter. In this experiment, therefore, the dextrose completely neutralized the inhibiting action of the nitrate upon the action of the bacterial secretions (*see* fig. 13, Plate 12).

TABLE VI—ACTION OF THE STERILE SECRETIONS OF NODULE BACTERIA UPON THE ROOT-HAIRS OF LUCERNE GROWN IN THE PRESENCE OF SODIUM NITRATE AND DEXTROSE

| Treatment | Number of replicate tubes | Estimates of root-hair deformation | | Counts of root-hairs in random microscope fields | |
|---|---|---|---|---|---|
| | | Total number of root-hairs examined | % root-hairs deformed | Total number of fields examined | Mean number of root-hairs per field |
| Control. No bacterial secretions; no nitrate or dextrose | 5 | 3220 | 12·6 ± 3·79 | 177 | 18·9 ± 2·41 |
| + bacterial secretions; no nitrate or dextrose ........ | 5 | 3104 | 83·1 ± 2·48 | 63 | 49·8 ± 3·80 |
| + bacterial secretions + 0·1% sodium nitrate ............ | 5 | 2980 | 42·5 ± 3·28 | 193 | 15·9 ± 2·30 |
| + bacterial secretions + 0·1% sodium nitrate and 0·5% dextrose ................ | 4 | 1701 | 61·3 ± 9·02 | 155 | 10·8 ± 2·37 |

TABLE VII—ACTION OF STERILE SECRETIONS OF NODULE BACTERIA UPON THE ROOT-HAIRS OF LUCERNE IN THE PRESENCE OF NITRATE AND DEXTROSE

| Treatment | Number of replicate tubes | Total number of root-hairs examined | % deformed root-hairs | Mean lengths in microns of 80 random root-hairs |
|---|---|---|---|---|
| + bacterial secretions. No nitrate or dextrose .................... | 4 | 893 | 48·97 ± 6·97 | 295·8 ± 11·5 |
| + bacterial secretions + 0·1% sodium nitrate ................ | 4 | 699 | 11·20 ± 1·32 | 156·8 ± 5·6 |
| + bacterial secretions + 0·1% sodium nitrate and 0·5% dextrose | 4 | 851 | 44·49 ± 4·81 | 290·0 ± 25·4 |
| No secretions, no nitrate or dextrose | 8 | 2616 | 6·16 ± 0·79 | 140·68 ± 13·3 |

These results support the view that the effect of nitrate is causally connected with a reduction in the carbohydrate available to the plant roots or at any rate with the ratio of these carbohydrates to nitrate.

## 7—THE MECHANISM OF THE NITRATE EFFECT UPON ROOT-HAIR DEFORMATION

In attempting to explain the mechanism by which nitrate protects the root-hairs against deformation by the bacterial secretions, it is necessary first to consider more closely the action of these secretions. There is evidence that they not only produce a deformed growth of the root-hairs but also increase their number and mean length.

That the presence of the secretions is accompanied by the increase in the number of the root-hairs is shown in Table VI, where the last column shows the results of root-hair counts from random microscope fields. In the series without secretions the mean number of root-hairs per microscope field was 18·9. This number was increased to 49·8 in the set receiving bacterial secretions but having otherwise similar treatment. The last column of Table III shows the similar effect of living bacteria.

The effect of secretions upon the length of root-hairs is shown in the last column of Table VII, which gives the mean lengths of 80 root-hairs taken at random in each of the different sets. A comparison with the first and the last set in this column shows a significant increase in root-hair length in the presence of the secretions, even though the lengths of the hairs in the presence of the secretions was doubtless under-estimated owing to the difficulty in measuring irregular root-hairs. Root-hairs are not usually deformed until they attain a certain length. Fig. 3 shows the frequency-distribution in length of 40 straight and 40 deformed root-hairs taken from the same six segments of a lucerne root grown in agar without nitrate in the presence of living nodule bacteria. There is rather sharp distinction in length between straight and deformed hairs on the same root.

Bacterial secretions thus exert an influence in stimulating the production and the growth in length of the root-hairs, as well as upon their deformation, and there is a close connexion between this increase in length and the deformation.

We may now consider the action of nitrate upon root-hair growth. We have already noted in § 4 the striking effect of nitrate in reducing the number of root-hairs that are produced in the presence of living bacteria, fig. 2. The similar effect of ammonium sulphate is shown in the last column of Table III. The normally stimulating action of bacterial secretions upon root-hair production is similarly checked by nitrate. This is shown by the counts set out in the last column of Table VI.

Nitrate also reduces the length which the root-hairs attain in the presence of nodule bacteria or their secretions. The data graphed in figs. 4 and 5 were obtained from material derived from the first experiment here described in § 4, where lucerne plants supplied with living nodule bacteria were grown in the presence and absence of nitrate. Fig. 4 shows the frequency-distribution in length of 200 root-hairs taken at random from six root segments out of the control set receiving no nitrate. The curve shown in fig. 5 was similarly obtained from the set receiving 0·5% sodium nitrate. The dots and crosses in both figures show, respectively, the relative numbers of straight and deformed hairs measured. In the presence of nitrate there is a greater frequency of root-hairs below the limit in length at which deformation normally occurs. The reduction in the percentage of deformed root-hairs can thus in part be accounted for by this checking in the growth of the hairs. The data graphed in these figures show, however, that in the nitrate set, very few deformed hairs occur even in the region of length, from 150 to 375 μ, at which deformation readily takes place in the control. The nitrate appears therefore to have an additional influence in checking the irregularity in the growth of the root-hairs that does take place.

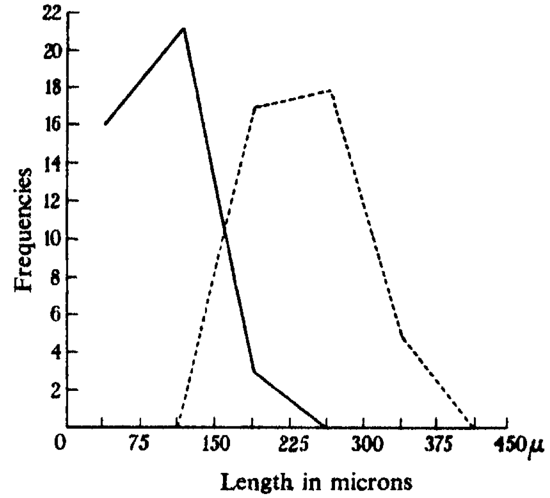


FIG. 3—Frequencies of root-hair lengths from 40 straight and 40 deformed root-hairs.
— straight ; --- deformed.

Evidence of the effect of nitrate upon root-hair length in the presence

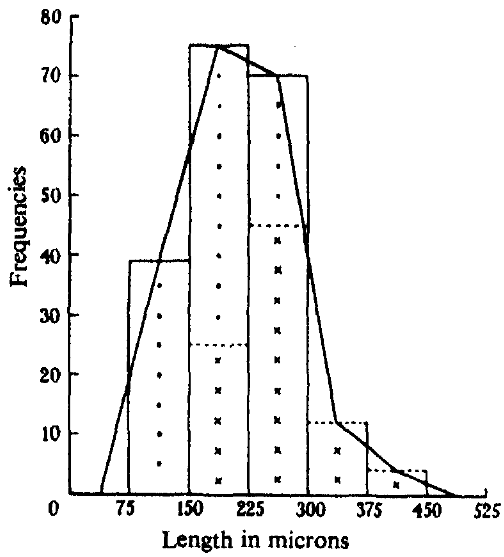


FIG. 4—Frequencies of root-hair length from 200 root-hairs on lucerne grown without nitrate. The dots and crosses indicate respectively the relative numbers of straight and deformed root-hairs at each range of length.

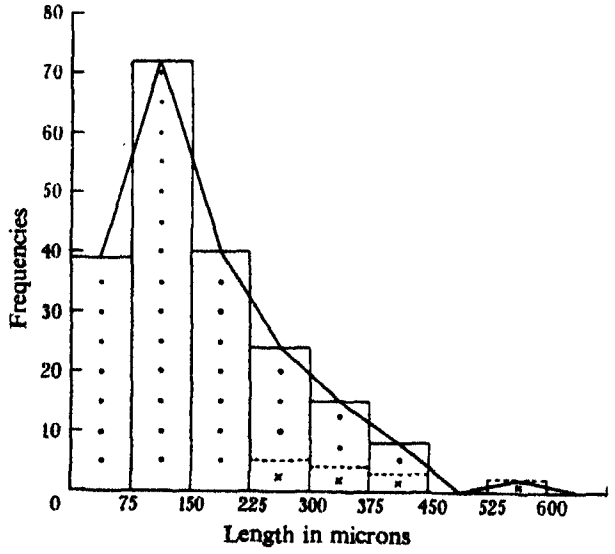


FIG. 5—Frequencies of root-hair length from 200 root hairs on lucerne grown with 0·5% sodium nitrate. The dots and crosses indicate respectively the relative numbers of straight and deformed root-hairs at each range of length.

of sterile bacterial secretions is shown in the last column of Table VII. Here the effect of treatments on root-hair lengths was similar to their influence on deformation.

We see then that the secretions of the nodule bacteria (*a*) increase the number of root-hairs, (*b*) stimulate their growth in length, and (*c*) produce irregular root-hair growth, these three effects being closely connected. Nitrate not only tends to stop this deformation of the root-hairs but also checks the increase in their number and lengths normally induced by the bacterial secretions. It seems reasonable therefore to connect the effect of nitrate in checking root-hair deformation with its general inhibitory action upon root-hair growth.

There is evidence suggesting that this checking in root-hair growth is due to a shortage of available carbohydrate or to a displaced equilibrium of carbohydrate to nitrogen in the epidermal cells of the root.

The author wishes to acknowledge the help given by Dr. Hugh Nicol in the course of the work here described.

## SUMMARY AND ABSTRACT

The actions of living nodule bacteria and of sterile filtrates containing their secretions were tested upon root-hairs of lucerne grown in agar medium in the presence and absence of sodium nitrate and ammonium sulphate.

Sodium nitrate at initial concentrations of from 0·1 to 1·0% prevents infection of the root-hairs.

Sodium nitrate at the above concentrations, and ammonium sulphate at a concentration of 0·1%, check the deformation of the root-hairs by the nodule bacteria. This deformation is a necessary prelude to infection and the checking of it accounts for the absence of infection by the nodule bacteria.

Sodium nitrate at a concentration of 0·1% also checks the deformation of root-hairs by sterile secretions of the nodule bacteria.

Both the living bacteria and their sterile secretions not only cause deformed growth of the root-hairs but in addition stimulate an increase in their number and length. Sodium nitrate also checks this growth stimulation.

These effects of nitrate in inhibiting the action of the bacterial secretions upon root-hairs are mitigated by the addition of dextrose, together with the nitrate, to the medium surrounding the roots. This suggests that the nitrate interferes with the carbohydrate supply to the piliferous layer of the root.

## DESCRIPTION OF PLATES

*Note*—All the photographs shown in both plates were made from lucerne roots stained *intra vitam* with aqueous methylene blue.

### PLATE 11

FIG. 6—Root-hair tip with colony of lucerne nodule bacteria, showing the bulging of the underlying cell-wall.

FIG. 7—Infection thread growing down the interior of a deformed root-hair.

FIG. 8—Sterile root grown in agar, showing straight root-hairs.

FIG. 9—Deformed root-hairs grown in agar without nitrate, but supplied with living lucerne nodule bacteria.

### PLATE 12

FIG. 10—Root-hairs grown in agar containing 0·1% of sodium nitrate and supplied with living lucerne nodule bacteria.

FIG. 11—Root-hairs grown in agar without nitrate and supplied with sterile bacterial secretions.

FIG. 12—Root-hairs grown in agar with 0·1% sodium nitrate and supplied with sterile bacterial secretions.

FIG. 13—Root-hairs grown in agar with 0·1% sodium nitrate, *plus* 0·5% dextrose and supplied with sterile bacterial secretions.

## REFERENCES

Allison, F. E., and Ludwig, C. A. (1934). ‘ Soil Sci.,’ vol. **37**, p. 431.
Flamand, H. (1903–4). ‘ L'Ingén. agric. Gembl.,’ vol. **14**, p. 755.
Fred, E. B., Baldwin, I. L., and McCoy, E. (1932). ‘ Univ. Wis. Stud. Sci.,’ No. 5.
Fred, E. B., and Graul, E. J. (1916). ‘ J. Amer. Soc. Agron.,’ vol. **8**, p. 316.
Fred, E. B., and Wilson, P. W. (1934). ‘ Proc. Nat. Acad. Sci. Wash.,’ vol. **20**, p. 403.
Giöbel, G. (1926). ‘ Bull. N.J. Agric. Expt. Stat.,’ No. 436.
Hills, T. L. (1918). ‘ J. Agric. Res.,’ vol. **12**, p. 183.
Hiltner, F. (1900). ‘ Arb. GesundhAmt., Biol. Abt.,’ vol. **1**, p. 177.
McCoy, E. (1932). ‘ Proc. Roy. Soc.,’ B, vol. **110**, p. 514.
Marchal, E. (1901). ‘ C.R. Acad. Sci. Paris,’ vol. **133**, p. 1032.
Müller, A., and Stapp, C. (1925). ‘ Arb. Biol. Abt. (Anst-Reichsanst.), vol. **14**, p. 455.
Ohkawara, S. (1928). ‘ Proc. 1st Int. Cong. Soil. Sci. Wash.,’ vol. **3**, p. 172.
Orcutt, F. S., and Wilson, P. W. (1934). ‘ Soil Sci.,’ vol. **39**, p. 289.
Strowd, W. H. (1920). ‘ Soil Sci.,’ vol. **10**, p. 343.
Thornton, H. G., and Nicol, Hugh (1935). ‘ J. Agric. Sci.’ (*In the press*).
Wilson, J. K. (1917). ‘ Bull. N.Y. (Cornell) Agric. Expt. Stat.,’ No. 386.

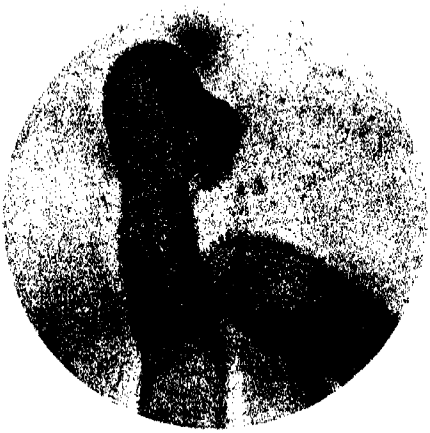


FIG. 6.

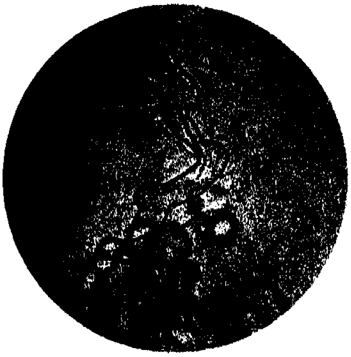


FIG. 7.

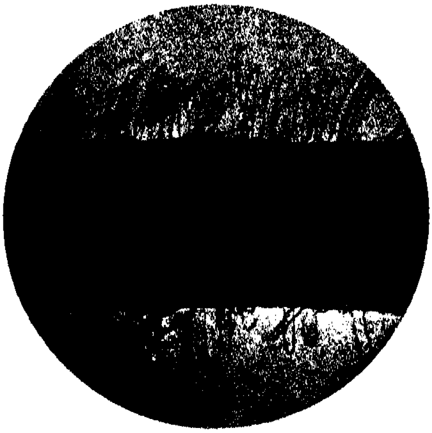


FIG. 8.

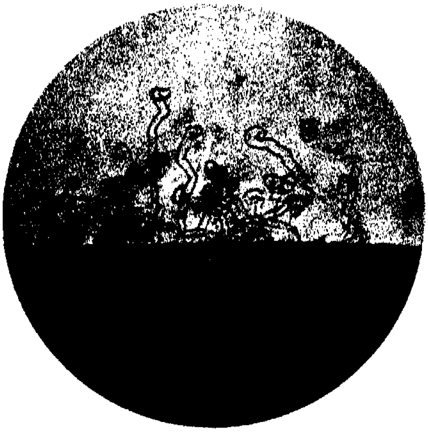


FIG. 9.

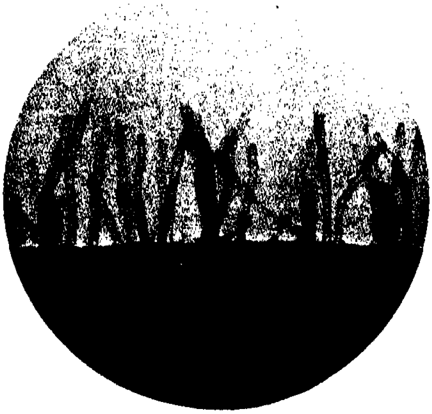


Fig. 10.

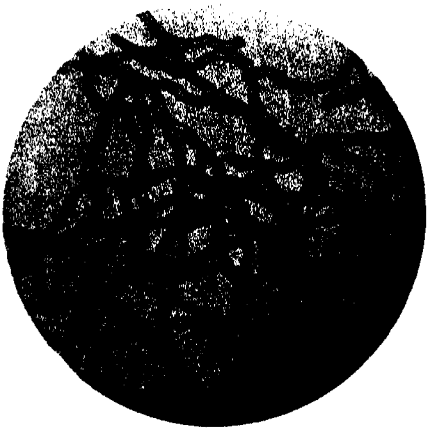


Fig. 11.

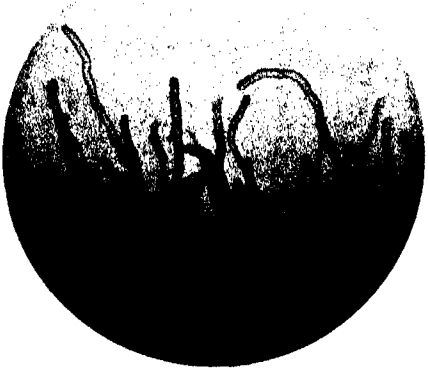


Fig. 12.

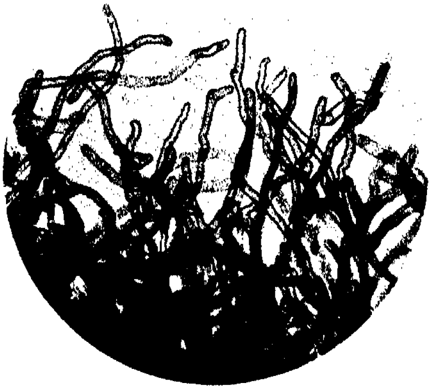


Fig. 13.

576.809.429:576.809.7

# Factors Affecting the Formation of Local Lesions by Tobacco Mosaic Virus

By JOHN CALDWELL, Department of Plant Pathology, Rothamsted Experimental Station*

(*Communicated by Sir John Russell, F.R.S.—Received November* 18, 1935)

[PLATE 13]

## INTRODUCTION

The increase in knowledge of the virus diseases of plants has led to the possibility of attempting an investigation of the nature of the virus agent itself. It has been proved that various plants, notably *N. glutinosa*, react to inoculation with the virus of tobacco mosaic by the formation of necrotic lesions on the rubbed leaves. It has been shown, further, that the number of lesions formed increases with the concentration of the virus up to a definite value, which varies with the experimental conditions (Caldwell, 1933). At high concentrations of the virus, there is apparently not a sufficient number of susceptible areas on the leaves to allow of reaction to all the virus units present, and the number of susceptible areas becomes the limiting factor in lesion formation. At lower concentrations the number of virus units is the main factor involved.

Youden, Beale, and Guthrie (1935) have recently published a paper in which they show that all the available data on the formation of lesions by different dilutions of virus, obtained by different workers, can be fitted to a curve with the formula $y = \mathrm{N}(1 - e^{-ax})$.

It has been shown that many substances, when added to the virus, have the effect of reducing the number of lesions on inoculation, or of completely suppressing lesion-formation. This may be due to the action of the substances on the virus agent itself or to their action on the tissues of the plants, whereby the normal response of the tissues to infection is prevented. It is not always clear which of the two effects is responsible for the suppression of the lesions in some instances. The purpose of the present paper is to set on record a method which, it is hoped, could be used in a chemical study of the nature of the virus agent, the agent not necessarily being freed from the plant materials with which it is associated. By the use of this method, it should be possible to determine whether an inhibitor is acting on the virus agent itself, or on the host tissues or on both more or less simultaneously.

---

* Now of the Department of Botany, University College, Exeter.

I have already shown that the curve indicating the number of lesions formed after rubbing leaves of *N. glutinosa* plants with different dilutions of the juice of tomato plants with aucuba mosaic has the form indicated in fig. 1.

In this the curve is at first more or less a straight line, while at the higher concentrations it tends to flatten out, as the number of susceptible areas becomes the limiting factor in the formation of lesions. The first part of the curve will the more closely approximate to a straight line with a slope of 1, the more nearly every virus unit (or a constant proportion of the units present) reaches a susceptible area of the leaf. This would occur if the method of inoculation should distribute the virus units sufficiently over the leaf-surface. As the concentration of virus increases, the ratio SA/V steadily falls (where SA = susceptible areas and V = the units of virus), until there are no longer any available susceptible areas and additional virus has no appreciable effect on the number of lesions.

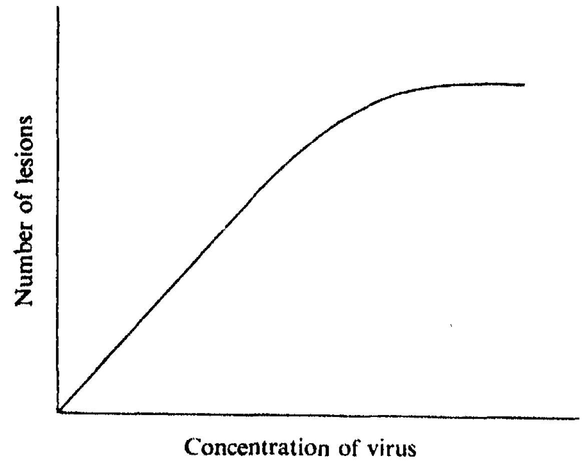


FIG. 1.

We have now to consider the position when a quantity of inhibitor is added which, by its action on the tissues of the leaf, reduces the number of susceptible areas to one-half, without affecting the virus agent. We assume that the same concentration of inhibitor is added to each concentration of virus. At the lowest concentration of virus, while the number of susceptible areas is reduced to one-half of that previously available, the ratio SA/V will still have a high value and the number of susceptible areas will be more than sufficient for the available virus units. The second curve will therefore coincide with the first in the early stages. As the concentration of virus increases, however, the ratio SA/V will decrease more rapidly in this instance and the curve will become horizontal sooner than did that for untreated virus, the values on the horizontal portion of the curve being one-half those on the same portion of the curve for normal virus material.

As can be seen from the curves in fig. 2 the values on the curve for treated virus will never be less than half those for the untreated virus, and will vary from equality to one-half.

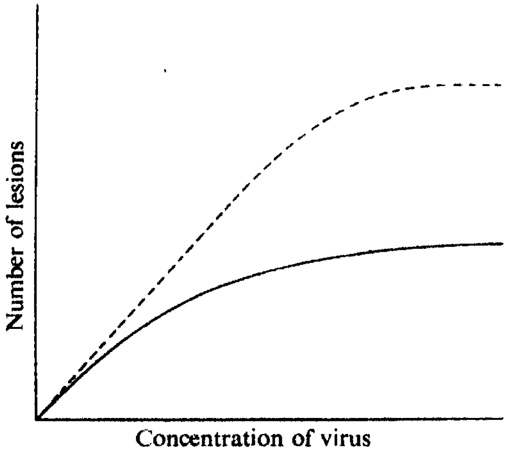


Fig. 2.

If the substance added to the virus reduces the number of lesions formed by its action on the virus agent itself and has no appreciable effect on the tissues of the host plant, then the curve for treated virus at different dilutions will take the form illustrated in fig. 3.

Here it is assumed that the inhibitor, under the conditions of the experiment, inactivates all the virus units at the lower concentrations. At these concentrations, therefore, no lesions are formed. As the concentration of virus increases, however, and the amount of inhibitor remains constant, the inhibitor will become saturated and an increasing amount of virus become available for lesion formation. The second curve, therefore, cuts the abscissa at some distance from the origin, and the distance between the point of intersection and the origin would increase with greater amounts of the inhibitor. As the concentration of the virus increases beyond the concentration at which the maximum number of lesions is obtained, then the effect of the inhibitor would cease to be evident, and the curve for treated virus would coincide with that for control virus.

It should be possible where the effect of an inhibitor is well marked to demonstrate whether its effect is on the virus or on the plant tissues. When the inhibitor inactivates the virus, its effect should increase with decreasing virus concentration, the amount of the inhibitor being kept constant. When the inhibitor affects the plant tissues, however, the effects should decrease with decreasing virus concentrations.

This is in agreement with the views of Youden, Beale, and Guthrie (1935) that the data shown on fig. 3 of their paper indicate an effect of the inhibitor on the virus and not primarily on the host. A host effect, as they suggest, would have given a curve parallel to the abscissa.

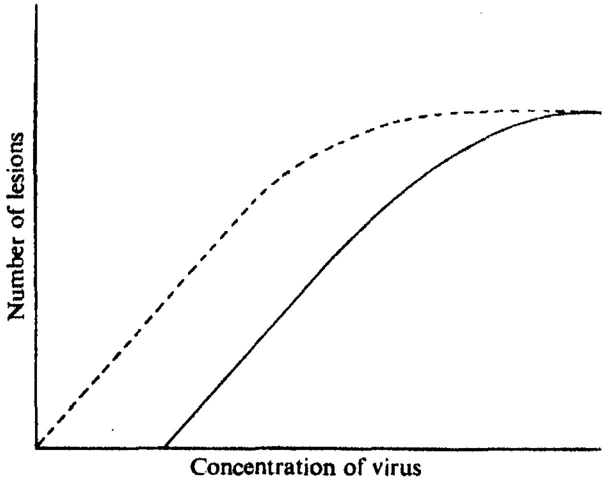


FIG. 3.

## MATERIALS AND METHODS

The viruses used in the investigations discussed in this paper were those of aucuba mosaic and of ordinary mosaic of the tomato. The former virus is similar to, if not identical with, Johnson's *No.* 6 *virus*, while the latter is *virus No.* 1 of Johnson (1927). In the main, the work was carried out in the laboratories of this Institute, but some of it was done in the laboratories of the Rockefeller Institute, Princeton, New Jersey, during a visit there in the spring of 1935.

The virus material was sometimes prepared by the methods outlined in earlier papers of this series, *i.e.*, the infected material was macerated with twice its weight of water, filtered through muslin and then through filter paper impregnated with fuller's earth. Crude juice from diseased plants was first frozen and subsequently filtered and used as the virus material, or in other instances the virus material was purified by the method of Vinson and Petre, as modified by the present author (1933). In a recent paper Petre (1935) supports the view that the modification increases the efficiency of the method. The method consists essentially in the precipitation of the plant proteins and of the virus agent itself by the use of lead acetate and the differential elution of the virus agent by suspension of the precipitate in M/15 di-potassium hydrogen phosphate. Where this material is used mention is made of the fact—and it was used primarily in experiments where only the minimum content was desirable.

For the preparation of virus material juice of diseased plants of the tomato *Lycopersicum esculentum* var. " Kondine Red ", of the tobacco *N. tabacum* var. " White Burley ", or, in America, of the tobacco variety " Turkish " was used. The inoculations for the assessment of virus content were made into leaves of *N. glutinosa* or into the cotyledons of the French bean *Phaseolus vulgaris*, var. " Golden Cluster ". The *N. glutinosa* plants were cut down to five similar adult leaves and inoculations of treated material were made into one-half of each leaf. Into the other half control material was inoculated and this gave a measure of the susceptibility of the leaves which inevitably vary considerably in their reaction to rubbing. By using the proportion between the number of lesions on the one-half as against those on the other, a more accurate measure was obtained, it is suggested, than would have been available had the actual numbers been taken. Inoculations made on one-half leaves with the other half as controls, obviated the necessity of using measured doses of virus material, as each of the half-leaves similarly treated, would presumably be equally inoculated. In any method of lesion counting for assessing the number of virus units, the volumes of the inoculated materials must be fairly constant. Inoculations were made by rubbing the leaves with the tip of the index finger, or by the use of a small pad of muslin which had been saturated in the material to be examined.

## PREVIOUS WORK ON THE EFFECT OF INACTIVATING SUBSTANCES ON THE VIRUS AGENT

Various workers have examined the effect of enzymes on plant and animal viruses and the results have, occasionally, been contradictory. Lojkin and Vinson (1931) found, for example, that none of the enzyme preparations which they used had the effect of inactivating the virus of tobacco mosaic—(*T.V. No.* 1)—a rather surprising result. Emulsin, pepsin, and yeast extract did not, they conclude, reduce the infectivity of the virus agent in purified preparations, while trypsin, pancreatin, and papain did. Pepsin apparently was effective after an incubation period of several days. The present author has shown (1933) that pepsin in low concentrations, added to virus juice at a $p_H$ of about 6·0 was ineffective. Pepsin at the same $p_H$, in high concentrations (*e.g.*, 2·5%) apparently does reduce the number of lesions formed on inoculation into *N. glutinosa* leaves (1935). This may be due in part to a protein effect such as is noted below. At a $p_H$ of 2·5, however, pepsin at a concentration of 1% commercial pepsin preparation completely destroys the activity of the agent of aucuba mosaic if kept in an incubator at 35° C for 48 hours. When the $p_H$ is returned to 6·0 and inoculations are made into *N. glutinosa* leaves no lesions are formed, and since pepsin is inactive at that $p_H$, and has been shown not to affect lesion formation (*see above*) it is fair to conclude that the virus agent has, in fact, been destroyed by the enzyme, since controls, held at the same temperature and at the same $p_H$, are almost equally effective before and after treatment. This is in accord with the data of Stanley (1934).

Lojkin and Vinson found that the enzyme effects were destroyed by boiling. They considered that the effect of trypsin, which was especially active in reducing the virus infectivity, was due to its hydrolytic action on the virus, rather than to a temporary inactivation of the virus by the enzyme. The evidence they adduce in support of the view is not very convincing. They exposed various virus preparations to trypsin at room temperature for periods varying from 1 hour to 22 days and found that these treatments reduced the activity of the virus agent to a greater or less extent. They then exposed similar juice to trypsin for 30 minutes at 0° C and found a reduction in the infectivity of almost 25%, which they considered as not significant. It must be noted, in this connexion, that these authors give surprisingly low infection percentages for their control plants inoculated with undigested juice. In all their experiments less than 100% infections of the inoculated tobacco plants was observed, when inoculated with the purified juice to which no enzyme was added, and the number of lesions formed on the leaves of *N. glutinosa* plants by similar material was of the order of 10–20. This would suggest that the methods of purification were not very satisfactory, since plant juice prepared by the method outlined in the papers of this series induces the formation of many hundreds of lesions on the inoculated leaves of *N. glutinosa*.

The general inference that Lojkin and Vinson drew from their results was that the enzymes acted on the virus as they would on protein substances, in other words, the effect on the virus was proteolytic.

In a paper published in 1933 I showed that the effect of trypsin on the virus was not proteolytic and that heating the trypsin-virus mixture at 70° C for 15 minutes, destroyed the enzyme, and resulted, on inoculation into the leaves of *N. glutinosa*, in the formation of necrotic lesions in approximately as large numbers as were induced by the original virus material. In a subsequent note Vinson (1934) has expressed his agreement with this conclusion. I attributed the absence of lesions after inoculation into the leaves of *N. glutinosa* to the inactivation of the virus, probably by adsorption on to the enzyme, and to the proteolytic action of the enzyme on the leaf tissues of the inoculated plants. No evidence was obtained that the enzyme, even in concentrations much greater than those used in the experiments with virus juice had any toxic effect on the tissues of the rubbed leaves. In a recent paper Stanley (1934) has concurred with the view that the effect of the enzyme on virus is not proteolytic. With some of my conclusions he has, however, disagreed and a subsequent section of this paper deals specifically with a discussion of the problem. The main conclusions at which Stanley arrived are as follows:

" If the virus were actually split or digested by trypsin, as first suggested by Vinson, reactivation by heat would be improbable . . . if the inactivation were due to proteolysis one would expect pepsin rather than trypsin to be an active proteolytic agent. . . . Since the virus in the trypsin-pepsin digest gave an average of about 77% as many lesions as did the virus in the untreated control solution, it is obvious that much of the virus actually was regained by digestion of the trypsin with pepsin." (This confirms the conclusion of my earlier paper.) . . . " If the loss of virus activity on the addition of trypsin is due to proteolysis it should take place only at hydrogen-ion concentrations at which trypsin is active . . . Trypsin has its maximum proteolytic activity between $p_H$ 7·5 and 9·5 and is inactive below 6·0." (This argument might equally well be used against the author's own conclusion that " trypsin . . . present at the time of inoculation or within 30 minutes thereafter, apparently affects infected cells, possibly by killing them " since the cell-sap of *N. glutinosa* leaves has a $p_H$ of the order of 5·5, . . . definitely on the acid side.) Stanley's conclusion is that " the effect of trypsin in causing a loss in virus infectivity is due chiefly to its action on the plant ".

The data that Stanley adduced on the diffusion rate of trypsin are probably not of much real value in a discussion on the existence of a virus-trypsin complex as it is arguable, as Stanley himself suggests, that the amount of trypsin was so much greater than the amount of virus present that the residual trypsin (assuming the existence of the complex) was insufficient in quantity to allow of its being considered as acting alone in the diffusion system. Stanley's own data published in Table VII of his paper suggest that the inactivation of the virus does enormously decrease with dilution, as tested by him on *P. vulgaris* leaves and that this is good presumptive evidence of an effect of the trypsin on the virus. At the same time it is clear that his data with *N. glutinosa* suggest that some effect on the plant-tissues is evident. In effect, Stanley has definitely confirmed my finding that the effect of trypsin on the virus is not proteolytic and that there is some effect on the virus, as opposed to the effect on the plant tissues. Which of these two effects is the more important, seems to be still a matter for discussion.

## EXPERIMENTS ON THE INACTIVATORY EFFECT OF NORMAL SERUM

The preparation of immune-sera by various workers (Beale, 1934; Birkeland, 1934; Chester, 1934, 1935) has greatly facilitated the separation and the identification of viruses by serological methods. The use of these sera, as specifically diagnostic material, has to some extent obscured the fact that many protein materials have at various times been used as inactivators of virus. It has been reported by various workers that the addition of proteins and similar substances has resulted in the reduction in number of spots following inoculation of a virus suspension into *N. glutinosa.* With a view to ascertaining whether the effect of the protein was primarily on the virus or on the plant tissues a series of experiments was carried out. In the earlier experiments, it was shown that 1% casein (soluble), 1% albumen, and 1% peptone in the virus suspension were effective in reducing the number of lesions to a greater or less extent. These substances were added to the virus juice and the mixture was left overnight on the laboratory bench before being used as inoculum. Actually, it is possible, as is described below, to inhibit the formation of lesions by the treatment of the leaves after inoculation with some of these and other substances.

After the preliminary experiments of a general nature, series of experiments were set up to ascertain whether or not the effect of the native proteins was primarily on the virus, or on the plant, as judged by the different effects with different concentrations of virus.

In most of the earlier work recorded in previous papers of this series whole leaves of plants of *N. glutinosa* were inoculated by rubbing, with the tip of the index finger, equal volumes of virus suspension on to each leaf. It was found that there was considerable variation from plant to plant and even between successive leaves on the same plant. The underlying causes of these differences are obscure but it is clear that they must be associated with the individual leaves. In an attempt to obviate the difficulties which arise by reason of these differences half-leaves were rubbed with the virus juice and were considered separately. It was found that in the main the right-halves of the leaves developed rather more lesions than did the left-halves, presumably because of the different treatment to which they, being more easily rubbed, were subjected. The difference might be as great as to give 75% the number of lesions on the left-halves that were developed on the right-halves. It was, therefore, found desirable to alternate the halves which were inoculated with juice after any one treatment and to use the other half-leaves for inoculation with the control solution. In this way, the following technique was evolved. The *N. glutinosa* plants were selected as soon as they had developed five mature adult leaves of approximately equal size, and the other leaves were removed. Thereafter a notch was cut in one or other side of each leaf, alternately on the left- and right-halves. Inoculations were made, with the untreated material on the un-notched side of each leaf, while on the other side, the corresponding material after treatment was applied. Thereafter, the *proportion* between the numbers of lesions induced by the treated as against the untreated was taken as a measure of the effect of the treatment.

In the first experiment the whole leaf was rubbed with virus material—juice of infected Turkish tobacco plants infected with tobacco *virus No.* 1 (*T.V. No.* 1). The tissues were macerated with twice the volume of water in the usual way and cleared by alternate freezing and thawing for a few days. The supernatant liquid was used as a source of material. This work was carried out at the Rockefeller Institute at Princeton, N.J. After the whole leaf had been rubbed one-half of each leaf was coated with normal rabbit serum, applied immediately with a camel-hair brush. There was a reduction in the number of the lesions on the treated half-leaves to about 40% of those on the untreated halves. In the next experiments the normal rabbit serum was mixed with an equal quantity of virus juice and similar juice was diluted to one-half with water to act as the control material. The mixtures were left on the laboratory bench for 2 hours and were then kept overnight frozen in a refrigerator. Inoculations were made by rubbing the juices on to half-leaves, using pads of cheese cloth soaked in the materials. No lesions whatsoever appeared on the half-leaves rubbed with the juice containing the 50% serum, while large numbers of lesions appeared on the other half-leaves. In another experiment only 2% normal rabbit serum was added to the virus juice, with appropriate controls, to which 2% extra water was added. In this case, the reduction in the number of lesions was approximately 50% of those developed on the control half-leaves.

It was clear from these experiments that 2% normal rabbit serum was rather too low a concentration to give complete inhibition in the use of crudely purified virus juice under the condition of the experiment. In the next experiment 10% of normal rabbit serum was added to diseased tobacco juice diluted to 1/5, 1/25, 1/125, 1/625, and to undiluted juice. After being kept overnight in a refrigerator, the juice was inoculated on to the half-leaves of *N. glutinosa* plants, when it was found that the inhibition was approximately 60%, 90%, 95%, 100%, and 100% in the dilutions 1/1, 1/5, 1/25, 1/125, 1/625, respectively. From the data, it was clear that increased dilution was associated with increased inhibition, with constant amounts of inhibitor, indicating that the effect of the inhibitor (some component of the normal rabbit serum) was in this instance acting primarily on the virus, if the argument set out in the earlier part of this paper be valid. It was noticed that there was some precipitate thrown down in those tubes which contained the higher concentrations of virus. To obviate so far as possible the removal of the virus mechanically by this precipitate, healthy tobacco juice was added to the serum before the diluted serum was mixed with the virus-containing material. Since the protein content of this healthy virus juice was far in excess of the much diluted virus juice, the formation of a precipitate between the serum and the plant-substances present in the virus material was largely or entirely prevented. Actually, cleared juice, prepared by filtration through fuller's earth or by the more elaborate method detailed above, rarely, and then only in the highest concentrations, gave any visible precipitate at all.

## EXPERIMENTS WITH HORSE SERUM

The preparation of normal rabbit serum involved a considerable amount of work, which was not entailed if normal horse serum produced commercially, was substituted for the normal rabbit serum. This was bought already prepared in 10 cc bottles.

The prepared serum contained traces of antiseptic substances (*e.g.*, tricresol) but there was no evidence, on comparison with normal rabbit serum, that this had any specific effect on either the virus or the tissues of the host plant.

In the preliminary experiment 1% normal horse serum was added to filtered juice from tomato plants with aucuba mosaic (strain Y) in concentrations of 1/1, 1/2, 1/4, 1/8, 1/16 and these were left overnight on the laboratory bench. Inoculations were made on the half-leaves of plants of *N. glutinosa*, using small muslin pads soaked in the treated juice or in the appropriate control juice. After inoculation, the leaves were washed with a stream of water to remove excess of the inoculum. In the course of the next few days lesions appeared on the treated plants, and it was found on the fifth day, that there was no recognizable inhibition in the three highest concentrations of juice (*i.e.*, 1/1, 1/2, 1/4) while there was an inhibition of lesion formation to the extent of 35% in the 1/8, and to 66% in the 1/16. This is, again, an instance of the effectiveness of a given concentration of serum being evident at low concentrations of virus, while in the presence of excess virus no evidence of inhibition was obtained. Since 1% normal horse serum had no evident effect on lesion formation at the higher concentrations of virus, the effect at lower concentrations could hardly be attributable to effects on the host tissues. The action of the serum must, therefore, be mainly on the virus itself. To establish this point a further experiment was set up in which 90% juice and 10% normal horse serum were tested against 90% juice and 10% healthy juice, and 10% juice, 10% serum, and 80% healthy tomato juice against 10% juice, 10% water, and 80% healthy tomato juice. 10% serum reduced the production of lesions by 90% virus juice by only 20% whereas the same concentration of serum completely inhibited the formation of lesions at the lower concentration of virus. The juice in this experiment was purified by the elution method, outlined above, and the M/15 di-potassium hydrogen phosphate eluate was adjusted to $p_H$ 7·2.

A large number of experiments has been carried out with aucuba mosaic juice, both purified and unpurified, and with larger or smaller quantities of serum added to different concentrations of the virus. To ensure that the protein content of the inoculum should be reasonably high the procedure has been to make up the serum or other test substance in double concentration in undiluted healthy tobacco juice, extracted and filtered through fuller's earth.

Invariably, with concentrations of serum around 5%, there was a progressively greater reduction in the proportion of the lesions induced by the untreated juice, as the concentration of the virus was reduced. It is abundantly clear, therefore, from these and from other experiments of Chester (1934) that normal serum does reduce the number of lesions induced on *N. glutinosa* and other hosts but it is suggested that the data here obtained and also those recorded by Chester indicate that the main action of the serum is on the virus itself.

## EXPERIMENTS WITH TRYPSIN

Similar experiments to those outlined above have been carried out with virus juice, prepared in various ways, to which small quantities of trypsin had been added. It was found in preliminary experiments that a 2% trypsin suspension washed over halves of leaves inoculated with virus juice greatly reduced the number of lesions on the treated as compared with the untreated half. This effect was most marked when the trypsin was applied immediately after inoculation when the lesions on the treated half-leaf were reduced to less than 10% of those on the other portion, and was progressively less marked as the interval between inoculation and treatment was increased to about an hour, when no reduction in lesion-number was noted. The reduction in the lesion-number when the trypsin-suspension was applied after a short period may be attributable to the effect of the enzyme on the injured tissue or on the virus agent itself, before the latter had become incorporated with the host tissue.

When 0·25% commercial trypsin (B.D.H.) was added to a series of dilutions of virus juice and the mixtures were kept at room temperature for 24 hours before inoculations were made as described for the serum-mixtures, the inhibition due to the trypsin did not show the marked effect of that due to the serum. At a dilution of 1/2, *i.e.*, with 50% healthy tobacco juice, and 0·05% trypsin the reduction in lesions on the treated half-leaves was 22% on an average, and at a dilution of 1/6 with the same enzyme concentration, 82%, while it rose to over 90% at 1/8, 1/54, and 1/162. This progressive increase in inhibition with decreased concentration of the virus has been shown to be indicative of an effect of the inhibitor on the agent, while the fact that the effect is not so pronounced as that of the serum, for example, would suggest that there is also an effect of the enzyme on the tissues. It is suggested, however, that while this effect is admitted, the fact that progressive increase in the inhibition with decreased concentration, which has been shown both by Stanley's experiments and by mine, indicate an effect of the trypsin on the virus, which as I have clearly shown, need not necessarily be proteolytic.

## EXPERIMENTS WITH SILVER NITRATE

A series of experiments has been carried out on the effect of the addition of silver nitrate on the production of lesions by aucuba mosaic. Chester (1935) has shown that silver nitrate does not appear to affect the antigenic properties of the virus of tobacco mosaic (T.M. *No.* 1). Various concentrations of virus material were made by extracting, under pressure, the juice of tomato plants infected with aucuba mosaic and then diluting the infected juice with expressed healthy juice. In this way it was hoped to keep the total amount of plant materials in the juice at about a constant level. The concentrations of virus were 1/2, 1/20, 1/200, 1/2000, and to each group were added constant amounts of silver nitrate to give concentrations of 0·1%, 1·0%, and 0·25%. The controls and the treated materials were allowed to stand for 24 hours on the laboratory bench and were then inoculated, as indicated above, the control material into one half-leaf and the corresponding treated material into the other. After a week the plants were examined when it was found that the silver nitrate had had the following effect on the formation of lesions.

In the series of dilutions to which 0·1% $AgNO_3$ had been added there was a slight reduction in only the 1/2000 concentration. This amounted to 40% on *an average of ten leaves* while in the higher concentrations of virus no effect of the silver nitrate was detectable. In the series to which 1·0% $AgNO_3$ was added the effect of the salt on the leaf-tissue was so pronounced that, even though the surplus inoculum in these, as in all the other experiments, was washed off with a stream of running water, the tissues were definitely affected by the inoculum and no virus necrotic lesions appeared on any of the treated plants at any concentrations of the virus.

In the third series the effect of the silver salt was very striking. In the lowest virus concentration there was a complete inhibition of lesion formation on the half of the leaves inoculated with the treated juice, while at 1/200 concentration only a single or at most a few lesions were formed the inhibition was more than 95% complete. At 1/20 concentration, however, considerable numbers of lesions were formed on the side inoculated with treated juice. The inhibition was of the order of 70%. In the highest concentration of virus, viz., 1/2 the lesions were so numerous on both sides of the leaf that it was impossible to demonstrate any inhibitory effect of the treatment at all. Four photographs showing the appearance of the treated leaves are given in Plate 13. In all these and similar experiments carried out with silver nitrate there was a copious precipitate in treated materials. This was thoroughly shaken up before inoculations were made and was inoculated on to the leaves under experimentation.

## DISCUSSION

It has been shown that the efficiency of an inhibitor acting on the virus itself should increase with decreasing concentration of virus while that of an inhibitor acting on the plant tissues should decrease with decreasing virus concentration. While no attempt is made to explain the action of the three substances used in the experiments, viz., trypsin, animal serum, and silver nitrate on the virus, it is suggested that the results obtained with them show that they have a definite effect on the virus present in the inocula.

No matter what that effect be due to, the method outlined in this paper, if used with substances the reactions of which on proteins and other substances are known, furnishes a means of studying the chemical nature of the virus agent, even if that agent were not necessarily in a pure state. The use of this method should make possible a direct chemical examination of the virus agent in an infected juice.

## SUMMARY

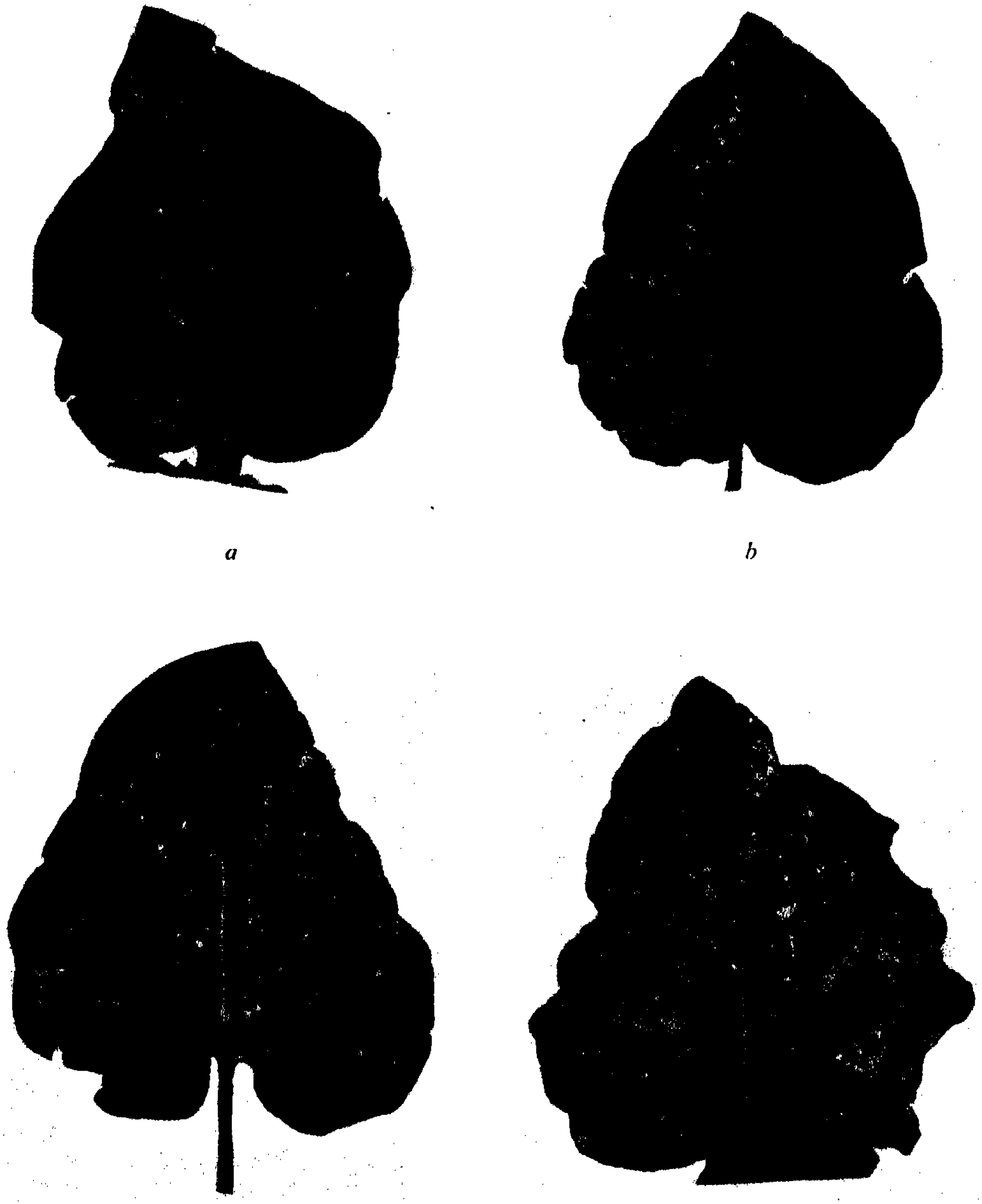
Many viruses produce local lesions when the plant juices containing them are rubbed on the leaves of appropriate plants. It is known that this effect may be reduced or even abolished by the addition of certain substances, *e.g.*, normal serum, to the virus-holding juice. The action of these inhibitory substances may be either directly on the virus itself, *e.g.*, by neutralizing its infectivity, or indirectly on the leaves of the rubbed plant, *e.g.*, by reducing their susceptibility; and it is a matter of some moment to distinguish between these two modes of action. A method is suggested whereby the distinction can be made.

The action of various enzymes on the virus activity is discussed, and in particular the effect of trypsin is studied experimentally, and shown to be in the main an action on the virus, not necessarily proteolytic. The inactivating effects of normal serum and of silver nitrate are studied, and found also to be due to action on the virus.

The use of the method described may make possible a direct chemical examination of the virus agent even in the presence of other constituents of the plant juice.

## REFERENCES

Beale, H. P. (1934). 'Contr. Boyce Thompson Inst.,' vol. **6**, p. 407.
Birkeland, J. M. (1934). 'Bot. Gaz.,' vol. **95**, p. 419.
Caldwell, J. (1933). 'Ann. Appl. Biol.,' vol. **20**, p. 100.
—— (1935). 'Ann. Appl. Biol.,' vol. **22**, p. 68.
Chester, K. S. (1934). 'Phytopath.,' vol. **24**, p. 1180.
—— (1935). *Ibid.*, vol. **25**, pp. 686 and 702.
Johnson, J. (1927). 'Wis. Agric. Expt. Sta. Res. Bull.,' vol. **76**



*a* *b*

*c* *d*

Leaves of plant of *N. glutinosa* inoculated with (*a*) $\frac{1}{2000}$, (*b*) $\frac{1}{200}$, (c) $\frac{1}{20}$, and (*d*) $\frac{1}{2}$ virus concentrations (Aucuba Mosaic) on half-leaves, and same concentrations with addition of 0·25% $AgNO_3$ on other half-leaves. (Photos. by V. Stansfield.)

Lojkin, M., and Vinson, C. G. (1931). 'Contr. Boyce Thompson Inst.,' vol. **2**, p. 147.
Petre, A. W. (1935). 'Contr. Boyce Thompson Inst.,' vol. **7**, p. 19.
Stanley, W. M. (1934). 'Phytopath.,' vol. **24**, pp. 18 and 1269.
Vinson, C. G. (1934). 'Science,' vol. **79**, p. 548.
Youden, W. J., Beale, H. P., and Guthrie, J. D. (1935). 'Contr. Boyce Thompson Inst.,' vol. **7**, p. 37.

581 : 12

# Researches on Plant Respiration

## IV—The Relation between the Respiration in Air and in Nitrogen of certain Seeds during Germination. (b) Seeds in which Carbohydrates Constitute the Chief Food Reserve

By William Leach

(*Communicated by W. Stiles, F.R.S.—Received November* 28, 1935)

### Introduction

Part III of this series (Leach and Dent, 1934) dealt with experimental data concerning the effect on the respiration rates and respiratory quotients of certain fat-storing germinating seeds when successively surrounded by atmospheres of air, nitrogen, and air. The present paper describes similar data relating to the respiration of germinating seeds in which the predominant food reserves are carbohydrates.

The seeds used in the present investigation, as with those used in the previous one, were of species belonging to genera widely separated from one another phyletically. The seeds were all obtained from Messrs. Sutton & Sons of Reading and the species and varieties used were as follows:—

*Fagopyrum esculentum*, *Zea mais* (Sutton's Improved Japanese Striped), and *Lathyrus odoratus* ("What Joy").

The seeds of all three species are similar in the respect that in each case starch forms the chief reserve food material.

The experimental data were all obtained by the katharometer method already described (Stiles and Leach, 1931; Leach, 1932) the experimental seedlings being maintained at a constant temperature of 25° C, and under other conditions identical with those used for *Ricinus*, *Helianthus*, and *Cucurbita*. Respiration rates are expressed as milligrams carbon-dioxide output per gram fresh (unsoaked) weight of seed (without seed coat) per hour.

## EXPERIMENTAL RESULTS

### 1—*Lathyrus odoratus*

The normal course of respiration, and the changes undergone by the respiratory quotient, during the early stages of germination of seeds of *Lathyrus odoratus* have been described and discussed at length in earlier papers of this series (Stiles and Leach, 1931, 1933). They are shown in fig. 1 which indicates the result of a recent experiment using three seeds with their testas previously removed. It will be noted that during the first 100 hours of germination, the output of carbon dioxide rises at a somewhat irregular rate to a maximum value, and having reached this maximum, it then begins to decrease. The respiratory quotient during this period appears to fluctuate somewhat but is, however, usually in the neighbourhood of 0·90.

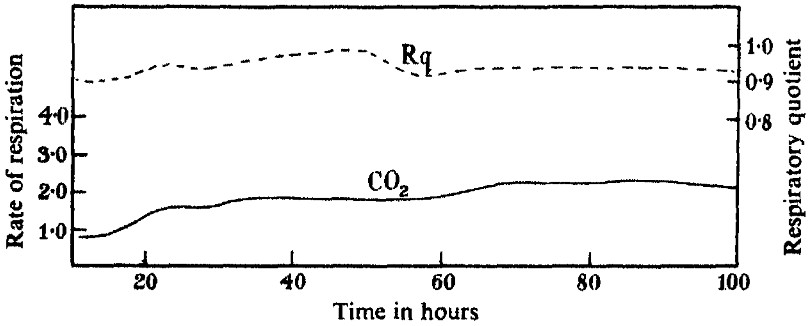


FIG. 1—*Lathyrus*, experiment 204.

The respiratory behaviour of the germinating seeds (without testas) of this species when the gas surrounding them is changed from air to nitrogen, and later from nitrogen back to air, is shown in Table I and fig. 2.

It will be observed from these that the change from air to nitrogen causes a rapid decrease in carbon dioxide output to an initial minimum rate. The value of this minimum rate as experimentally recorded varies between one-third and one-half of the rate recorded immediately before the air was replaced by nitrogen.

Following on this initial fall, the production of carbon dioxide increases for some hours until a maximum is reached, after which it again decreases, continuing to do so throughout the remainder of the period during which

TABLE I—THE RESPIRATORY BEHAVIOUR OF *Lathyrus odoratus* IN AIR AND IN NITROGEN

Experiment number

| 168<br>One seed used | | | 170<br>One seed used | | | 171<br>One seed used | | | 203<br>Three seeds used | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Age of seedling in hours | Respiration rate mg $CO_2$/gm/hour | Rq | Age of seedling in hours | Respiration rate mg $CO_2$/gm/hour | Rq | Age of seedling in hours | Respiration rate mg $CO_2$/gm/hour | Rq | Age of seedling in hours | Respiration rate mg $CO_2$/gm/hour | Rq |
| | Air | | | Air | | | Air | | | Air | |
| 58·5 | 1·08 | 0·81 | 69·0 | 1·11 | 0·87 | 100·5 | 2·71 | 0·91 | 56·0 | 1·21 | 0·88 |
| 64·5 | 1·18 | 0·81 | 87·0 | 1·16 | 0·88 | 105·5 | 2·41 | 0·91 | 62·0 | 1·14 | 0·89 |
| 71·0 | 1·18 | 0·84 | 90·5 | Nitrogen | | 110·5 | 2·16 | 0·90 | 68·0 | 1·06 | 0·89 |
| 76·0 | Nitrogen | | 92·0 | 0·72 | | 114·5 | Nitrogen | | 71·5 | Nitrogen | |
| 78·5 | 0·49 | | 93·0 | 0·39 | | 116·0 | 0·78 | | 72·5 | 0·76 | |
| 82·5 | 0·64 | | 94·0 | 0·27 | | 117·0 | 1·13 | | 74·0 | 0·58 | |
| 86·0 | 0·67 | | 96·5 | 0·45 | | 121·5 | 1·18 | | 76·0 | 0·55 | |
| 89·0 | 0·51 | | 102·5 | 0·50 | | 124·5 | 1·41 | | 78·0 | 0·49 | |
| 93·5 | Air | | 109·5 | 0·66 | | 128·5 | 1·48 | | 80·5 | 0·61 | |
| 95·5 | 1·09 | 0·82 | 115·0 | 0·59 | | 134·0 | 1·41 | | 83·5 | 0·65 | |
| 97·5 | 1·04 | 0·72 | 123·5 | 0·52 | | 144·0 | 1·11 | | 86·5 | 0·74 | |
| 100·5 | 1·08 | 0·68 | 135·5 | 0·54 | | 156·5 | 0·89 | | 89·5 | 0·65 | |
| 104·5 | 1·47 | 0·79 | 146·0 | 0·47 | | 167·0 | Air | | 93·0 | 0·67 | |
| 112·0 | 1·80 | 0·86 | 174·0 | 0·41 | | 169·0 | 2·44 | 0·88 | 94·0 | Air | |
| 119·5 | 1·42 | 0·85 | 182·5 | 0·37 | | 172·0 | 2·61 | 0·83 | 95·5 | 0·95 | 0·95 |
| 130·5 | 1·41 | 0·85 | 187·5 | Air | | 175·5 | 2·52 | 0·80 | 97·5 | 0·98 | 0·89 |
| | | | 190·5 | 1·13 | 0·70 | 181·0 | 2·24 | | 99·5 | 1·08 | 0·60 |
| | | | 192·5 | 1·24 | 0·70 | 189·5 | 2·89 | 0·94 | 101·5 | 0·98 | 0·72 |
| | | | 194·5 | 1·01 | 0·64 | 195·5 | 3·78 | 0·97 | 103·5 | 1·35 | 0·79 |
| | | | 196·5 | 1·19 | 0·67 | 201·0 | 3·67 | 0·97 | 106·5 | 1·39 | |
| | | | 200·5 | 1·35 | 0·69 | | | | 110·5 | 1·39 | 0·89 |
| | | | 205·5 | 1·50 | 0·70 | | | | 116·0 | 1·29 | |
| | | | 209·0 | 1·65 | 0·75 | | | | 121·0 | 1·10 | 0·89 |
| | | | 214·0 | 1·43 | 0·87 | | | | 125·0 | 1·04 | 0·88 |
| | | | 217·0 | 1·55 | 0·95 | | | | 129·0 | 1·06 | 0·89 |
| | | | 229·5 | 2·32 | 1·00 | | | | 133·0 | 1·12 | 0·90 |
| | | | | | | | | | 137·0 | 1·20 | 0·91 |

the seedlings are kept in nitrogen. The falling off in the rate of carbon dioxide production, from this maximum value in nitrogen, is slow and in only one of the experiments performed (namely experiment 170 in which the nitrogen period was prolonged to 90·5 hours) was its final recorded value lower than the initial minimum referred to above.

The change to respiration in air, after the period in nitrogen, results in a rapid rise in carbon dioxide output until this is approximately the same as it originally was before the change from air to nitrogen was made. A further and usually more gradual rise in carbon dioxide output then follows until a maximum value is reached after which the rate decreases for a time and then again increases. This final increase seems to be the settling down of the respiration curve to its normal course for seedlings respiring in air, the form of which is determined by the developmental stage in which the particular seedling happens to be.

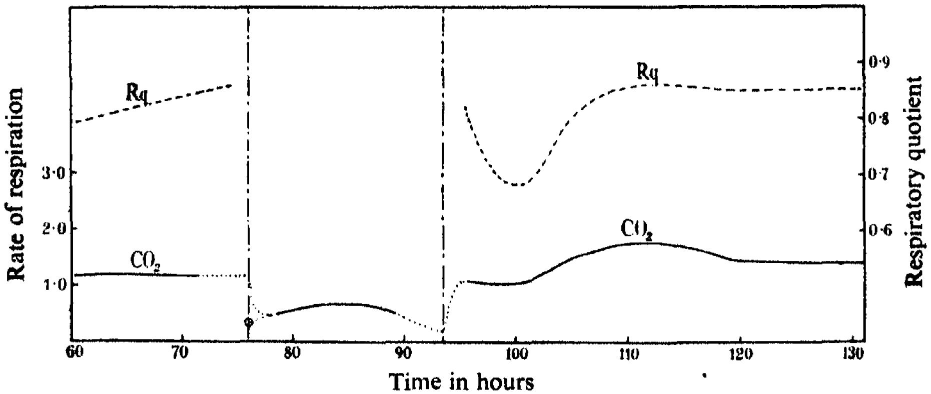


FIG. 2—*Lathyrus*, experiment 168.

The effect of the period in nitrogen on the respiratory quotients of the seedlings, when they are again respiring in air is similar to that found for fat-storing seeds. The change from anaerobic to aerobic conditions is followed by a relatively high rate of oxygen intake as compared with carbon dioxide output. The beginning of the second period in air is characterized by a falling respiratory quotient. This fall is continued for a period of time varying between 5 and 10 hours until a minimum value is reached. As will be seen from the data given in Table I this minimum value lies more commonly between 0·60 and 0·70 although in experiment 171 the lowest value recorded was only 0·80.

After reaching this minimum the respiratory quotient rises to a level approximating to unity and thus takes up its normal value for aerobic respiration.

### 2—*Fagopyrum esculentum*

The course of respiration and the changes taking place in the value of the respiratory quotient, during the early period of germination in air, of seeds of *Fagopyrum* are shown in fig. 3. From the start the carbon dioxide output increases at a comparatively rapid rate, while the respiratory quotient rises from an initially low value practically to unity (Stiles and Leach, 1933).

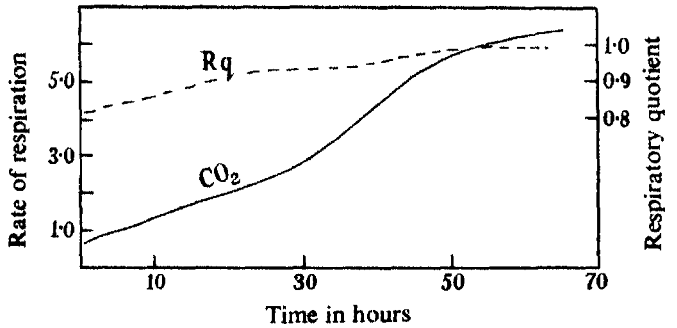


FIG. 3—*Fagopyrum*, experiment 119.

The interruption of this normal behaviour, by the introduction of a period of anaerobiosis in nitrogen, results in disturbances which show certain resemblances to, and certain differences from, those already described for *Lathyrus*.

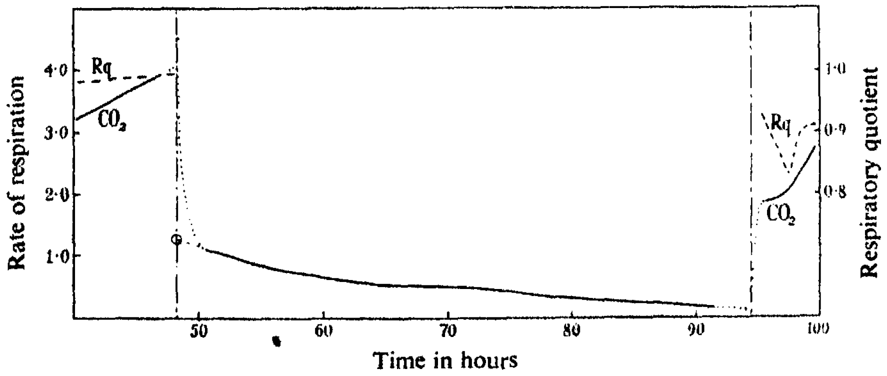


FIG 4—*Fagopyrum*, experiment 191.

Table II sets out the numerical details of four experiments performed on germinating seeds of *Fagopyrum*, and the results of one of these experiments are shown graphically in fig. 4.

An examination of these data shows that on changing from air to nitrogen, the usual rapid fall in carbon dioxide output takes place, this

TABLE II—THE RESPIRATORY BEHAVIOUR OF *Fagopyrum esculentum* SEEDLINGS IN AIR AND IN NITROGEN

| Experiment number | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 186<br>10 seeds used | | | 191<br>10 seeds used | | | 201<br>10 seeds used | | | 202<br>8 seeds used | | |
| Age of seedling in hours | Respiration rate mg $CO_2$/gm/hour | Rq | Age of seedling in hours | Respiration rate mg $CO_2$/gm/hour | Rq | Age of seedling in hours | Respiration rate mg $CO_2$/gm/hour | Rq | Age of seedling in hours | Respiration rate mg $CO_2$/gm/hour | Rq |
| | Air | | | Air | | | Air | | | Air | |
| 25·5 | 1·84 | 0·93 | 27·5 | 1·64 | 0·96 | 21·5 | 0·68 | 1·05 | 27·0 | 2·03 | 0·80 |
| 27·5 | 2·04 | 0·94 | 30·5 | 2·07 | 0·96 | 25·5 | 1·24 | 1·06 | 33·0 | 2·81 | 0·97 |
| 28·5 | Nitrogen | | 47·0 | 3·93 | 0·99 | 29·5 | 1·75 | 1·04 | 37·0 | 3·28 | 0·97 |
| 30·0 | 0·73 | | 48·25 | Nitrogen | | 34·5 | 1·85 | 1·05 | 39·0 | 3·66 | 0·97 |
| 32·0 | 0·70 | | 50·5 | 1·11 | | 39·0 | 2·22 | 1·02 | 41·5 | Nitrogen | |
| 34·0 | 0·57 | | 53·5 | 0·95 | | 42·25 | Nitrogen | | 43·0 | 1·40 | |
| 36·0 | 0·61 | | 56·5 | 0·74 | | 43·5 | 0·84 | | 45·0 | 1·17 | |
| 38·0 | 0·56 | | 59·5 | 0·69 | | 45·5 | 0·73 | | 47·0 | 1·03 | |
| 40·0 | 0·49 | | 62·5 | 0·57 | | 47·5 | 0·56 | | 49·0 | 0·91 | |
| 42·5 | 0·48 | | 65·0 | 0·51 | | 49·5 | 0·48 | | 51·0 | 0·86 | |
| 46·5 | 0·12 | | 72·5 | 0·50 | | 51·5 | 0·60 | | 53·0 | 1·03 | |
| 51·5 | 0·08 | | 75·5 | 0·39 | | 53·5 | 0·68 | | 55·0 | 0·95 | |
| 62·5 | 0·003 | | 78·5 | 0·33 | | 56·5 | 0·72 | | 58·0 | 0·82 | |
| 71·5 | Air | | 81·5 | 0·30 | | 60·5 | 0·66 | | 61·5 | 0·77 | |
| 73·0 | 1·24 | 0·87 | 84·5 | 0·25 | | 65·0 | 0·60 | | 63·25 | Air | |
| 75·0 | 1·26 | 0·78 | 87·5 | 0·20 | | 66·0 | Air | | 64·5 | 2·30 | 0·86 |
| 77·0 | 1·47 | 0·79 | 90·5 | 0·17 | | 67·5 | 2·10 | 0·80 | 66·5 | 3·00 | 0·91 |
| 81·0 | 1·69 | | 93·0 | 0·15 | | 69·5 | 2·50 | 0·93 | 68·5 | 3·69 | 0·95 |
| 97·5 | 1·57 | 0·93 | 94·5 | Air | | 71·5 | 2·57 | 0·96 | 70·5 | 3·64 | 0·96 |
| 100·0 | 1·24 | 0·99 | 95·5 | 1·85 | 0·92 | 73·5 | 2·57 | 0·97 | 73·0 | 4·11 | 0·97 |
| | | | 96·5 | 1·88 | 0·88 | 75·5 | 2·61 | 0·98 | 90·0 | 4·43 | 0·97 |
| | | | 97·5 | 2·03 | 0·83 | 77·5 | 2·78 | 0·98 | 93·5 | 4·29 | 0·97 |
| | | | 98·5 | 2·36 | 0·90 | 79·5 | 2·87 | 0·99 | 96·0 | 4·20 | 0·96 |
| | | | 99·5 | 2·71 | 0·91 | 81·5 | 2·88 | 1·00 | | | |
| | | | | | | 90·5 | 3·48 | | | | |
| | | | | | | 94·5 | 3·40 | 1·03 | | | |
| | | | | | | 98·0 | 3·70 | 1·03 | | | |

being followed by a slower rate of decrease. In two experiments, namely numbers 201 and 202, this decrease is followed by a very definite rise and a second fall similar to that observed with *Lathyrus odoratus*. In experiment 186 this rise which occurs between the 34th and 38th hours is only very slight, while in experiment 191 it was not recorded at all. No explanation can be offered to account for this difference between the behaviours of the seedlings used in these two pairs of experiments beyond that it may possibly be due to an after-ripening effect; experiments 186 and 191 were respectively carried out in February and March of 1934 and the seeds used were of the 1933 crop, whereas experiments 201 and 202 used seeds of the 1934 crop and were both carried out in November of that same year.

Seedlings of *Fagopyrum* on being restored to aerobic conditions after a period in nitrogen appear quickly to resume their normal aerobic respiratory behaviour. The change from nitrogen to air is marked by the usual rapid rise in carbon dioxide output which has been observed in the other species used in these investigations. This rise continues until the rate approaches that recorded at the time the change from air to nitrogen was made. A slower rise in carbon dioxide output follows as the normal aerobic course of respiration of the seedlings is gradually resumed. The definite rise to a maximum and subsequent temporary fall in the respiration rate at this stage of the experimental treatment, so pronounced in *Lathyrus*, is only shown very slightly in one of the experiments with *Fagopyrum* (namely experiment 202 after 68·5 hours).

The behaviour of the respiratory quotient on changing from nitrogen to air is essentially the same for *Fagopyrum* as was recorded for *Lathyrus*. It undergoes an initial rapid fall to a minimum value from which it rises and takes up the normal course it follows in seedlings respiring in air, being finally maintained at or very close to unity. Experiments 186 and 191 show a difference from experiments 201 and 202 in addition to that mentioned above. In the former, the fall in the respiratory quotient, immediately after the change from nitrogen to air, is much slower than in the latter, in fact in both experiments 201 and 202 it took place during a period of less than 1·5 hours and was consequently not recorded. In experiments 186 and 191 it extended over approximately 3·5 hours and 3·0 hours respectively.

### 3—*Zea mais*

Experimental data for *Zea mais* of a corresponding nature to those already given and dealt with for *Lathyrus* and *Fagopyrum* are set out in

Table III. The normal course of respiration and the changes in the respiratory quotient during the germination of a single seed of the variety used in these experiments are shown in fig. 5. The respiratory behaviour of a seedling under successive aerobic and anaerobic experimental conditions is shown in fig. 6.

Replacement of the air surrounding the seedlings by nitrogen produces a similar effect on their carbon dioxide output to that produced when *Lathyrus* seedlings are used. The rate of carbon dioxide output falls to a minimum value from which it rises to a maximum and then again falls.

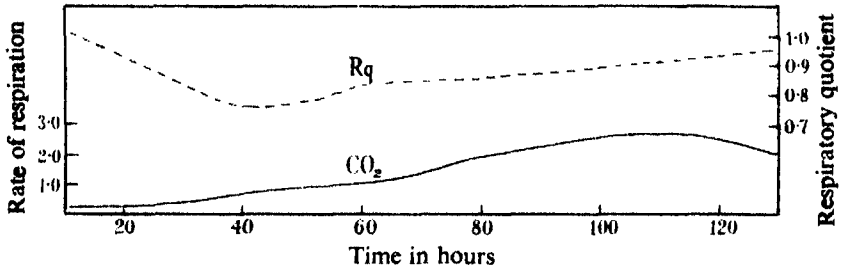


FIG. 5—*Zea mais*, experiment 192.

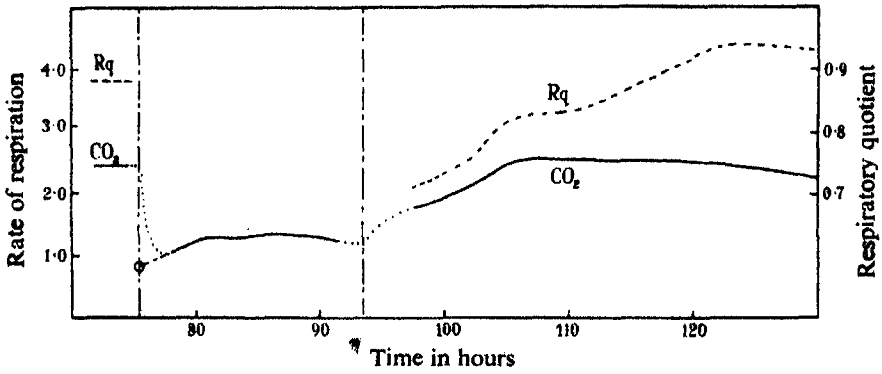


FIG. 6—*Zea mais*, experiment 181.

The change from nitrogen to air at the end of the experimental period in nitrogen results in a rapid rise in the carbon dioxide output which then more gradually assumes its normal aerobic course. At this stage of the treatment the behaviour of *Zea mais* resembles that of *Fagopyrum* rather than *Lathyrus* as no definite rise to a maximum and subsequent fall, before the resumption of the normal course, was recorded.

The behaviour of the respiratory quotient during these last aerobic stages presents no marked difference from what occurs in the other two

TABLE III—THE RESPIRATORY BEHAVIOUR OF *Zea mais* SEEDLINGS IN AIR AND IN NITROGEN

| Experiment number | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 177 One seed used | | | 178 One seed used | | | 181 One seed used | | | 205 Three seeds used | | |
| Age of seedling in hours | Respiration rate mg $CO_2$/gm/hour | Rq | Age of seedling in hours | Respiration rate mg $CO_2$/gm/hour | Rq | Age of seedling in hours | Respiration rate mg $CO_2$/gm/hour | Rq | Age of seedling in hours | Respiration rate mg $CO_2$/gm/hour | Rq |
| | Air | | | Air | | | Air | | | Air | |
| 37·5 | 0·72 | 0·87 | 73·0 | 2·44 | 0·88 | 73·5 | 1·91 | 0·82 | 54·5 | 0·62 | 0·82 |
| 41·5 | 0·80 | 0·84 | 75·5 | Nitrogen | | 76·0 | Nitrogen | | 60·5 | 0·63 | 0·81 |
| 48·5 | 0·76 | 0·86 | 78·0 | 1·04 | | 78·0 | 0·71 | | 65·5 | 0·82 | 0·86 |
| 52·0 | Nitrogen | | 80·5 | 1·29 | | 81·0 | 0·93 | | 68·0 | Nitrogen | |
| 54·5 | 0·55 | | 83·5 | 1·27 | | 84·5 | 0·91 | | 69·5 | 0·44 | |
| 56·5 | 0·55 | | 87·0 | 1·33 | | 88·5 | 1·07 | | 73·5 | 0·35 | |
| 59·0 | 0·72 | | 91·0 | 1·26 | | 93·0 | 0·90 | | 75·5 | 0·39 | |
| 63·0 | 0·70 | | 93·5 | Air | | 97·0 | Air | | 77·5 | 0·58 | |
| 67·0 | 0·70 | | 97·5 | 1·76 | 0·71 | 99·5 | 1·26 | 0·79 | 79·5 | 0·59 | |
| 71·5 | 0·63 | | 102·5 | 2·15 | 0·76 | 102·0 | 1·28 | 0·73 | 81·5 | 0·54 | |
| 75·5 | Air | | 105·5 | 2·52 | 0·82 | 105·0 | 1·58 | 0·80 | 85·5 | 0·51 | |
| 78·0 | 1·04 | | 109·5 | 2·54 | 0·83 | 108·0 | 1·69 | 0·82 | 87·5 | 0·54 | |
| 80·5 | 0·93 | 0·79 | 113·5 | 2·52 | | 111·0 | 1·80 | 0·82 | 89·5 | 0·55 | |
| 85·5 | 1·15 | 0·85 | 123·0 | 2·46 | 0·94 | 120·0 | 2·25 | 0·85 | 91·5 | 0·46 | |
| 90·5 | 1·23 | | 130·5 | 2·21 | 0·93 | 123·0 | 2·24 | 0·84 | 93·0 | 0·49 | |
| 96·0 | 1·24 | | 143·5 | 2·42 | 0·86 | 126·0 | 2·06 | 0·83 | 95·0 | Air | |
| | | | | | | 152·0 | 1·97 | 0·93 | 97·5 | 0·60 | 0·75 |
| | | | | | | | | | 102·0 | 0·95 | 0·79 |
| | | | | | | | | | 108·0 | 1·09 | 0·81 |
| | | | | | | | | | 114·0 | 1·07 | 0·83 |
| | | | | | | | | | 119·5 | 1·09 | 0·86 |

species dealt with in this paper. At the beginning of the final aerobic period the respiratory quotient falls rapidly to a relatively low value, after which it follows a somewhat irregular rising course until normal behaviour is resumed.

## DISCUSSION

The investigations described in this paper form a direct continuation of those described in Part III (Leach and Dent, 1934). It is not therefore proposed to restrict this discussion to a consideration of the experimental results obtained with carbohydrate-storing seeds only, but to deal briefly with both carbohydrate and fat-storing seeds.

The three starch-storing seeds, namely those of *Lathyrus*, *Zea*, and *Fagopyrum* show a very similar respiratory behaviour. In all three the change from respiration in air to respiration in nitrogen is marked by a rapid and considerable fall in the output of carbon dioxide. The initial low rate of anaerobic carbon dioxide production in all three seeds is followed by a rise and subsequent fall. This last fall, as shown in experiments 170 and 171 with *Lathyrus* and more definitely in experiments 186 and 191 with *Fagopyrum*, appears to continue throughout the period during which the seeds are kept without oxygen.

The change from anaerobic respiration in nitrogen to aerobic respiration, produces an immediate rise in the carbon dioxide output. In *Lathyrus* this rise continues to a maximum from which it then falls for a few hours, this fall being followed by a more gradual rise, as the normal aerobic rate of respiration of the seedling is gradually resumed. In *Zea* and *Fagopyrum* this early maximum does not occur when the change from nitrogen to air is made, instead, the carbon dioxide output merely increases until the respiration of the seedlings again follows its normal course.

The behaviour of the respiratory quotients in these three carbohydrate-storing seeds, when the change from anaerobic to aerobic conditions is made, corresponds to that observed with fat-storing seeds. The respiratory quotient first falls, usually rapidly, and then rises more gradually to its normal value for the particular seedling under observation. There is thus evidence that in carbohydrate-storing seeds also, some easily oxidizable substance accumulates during anaerobiosis, which, when oxygen is again supplied, is rapidly respired. In *Lathyrus* it would appear that this substance is sufficiently active, or accumulates in sufficient quantity, to cause the rate of carbon dioxide output to rise to a value that cannot be maintained, thus causing the peak in the respiration rate referred to above. It is of interest to find that Jensen (1923) records a similar temporary abnormal aerobic respiration rate for seedlings of *Sinapis alba* after they had been subjected to a period of anaerobiosis in hydrogen.

It will now be of some interest to consider in more detail the respiratory behaviour of the seedlings under investigation, when they are first brought from air into nitrogen. This behaviour in the case of apples was used by Blackman (1928) for the purpose of deducing their rates of glycolysis at the times when the changes from air to nitrogen were made. Therefore, using the graphs constructed from the data obtained from each of the experiments carried out in the present investigation, values were obtained by extrapolation for the rates of carbon dioxide output in air (OR) and in nitrogen (NR) at the moments when the changes from air to nitrogen were made. These values were used in the working out of the NR/OR ratios for each species examined and which are given in Table IV.

If the values of NR/OR for the three carbohydrate seeds are examined it will be noted that the highest figure obtained is 0·37 for *Fagopyrum*, experiment 202, while in the remaining experiments with this species and in the experiments with *Zea* and *Lathyrus* values for NR/OR range down to 0·17.

A point of considerable interest is the close approximation to 0·33 shown by NR/OR in all four experiments with *Fagopyrum*. From this it may be assumed that in *Fagopyrum* the output of carbon dioxide in nitrogen (NR) is one-third of the output in oxygen (OR) at the moment when the transition from oxygen to nitrogen occurs. In other words the change is exactly what would take place if the complete oxidation of hexose to carbon dioxide and water were replaced by its anaerobic breakdown to carbon dioxide and alcohol. In *Fagopyrum* seedlings then (using Blackman's terminology),

$$\text{Glycolysis (Gl)} = \text{Aerobic respiration (OR)}$$

and therefore no oxidative anabolism takes place.

Oxidative anabolism also appears to be absent from the respiratory processes of *Lathyrus* and *Zea*, as with neither of these species does NR/OR ever exceed 0·33. It is not clear why, in the seedlings of these latter, NR/OR shows such low values. The explanation may be that the values of NR adopted for these two seedlings are perhaps too low owing to the difficulty of making correct extrapolations. This is because of the complications in their respiration curves, caused by the pronounced rise in carbon dioxide production in nitrogen which follows the initial transitional fall.

A possible reason why, in *Fagopyrum*, the value of NR/OR so closely approximates to theoretical expectations, may be connected with the fact that the seed of this species is a relatively simple morphological type. It is small, non-endospermic with epigeal cotyledons; and the food store consists almost entirely of starch. When germination occurs practically the whole of the embryo is composed of growing tissues, and owing to its small size the diffusion paths of respiratory gases are short.

TABLE IV

| Seedling | Experiment No. | $\frac{NR}{OR}$ |
|---|---|---|
| *Lathyrus odoratus* | 168 | 0·25 |
| | 170 | 0·18 |
| | 171 | 0·25 |
| | 203 | 0·20 |
| *Fagopyrum esculentum* | 186 | 0·36 |
| | 191 | 0·31 |
| | 201 | 0·36 |
| | 202 | 0·37 |
| *Zea mais* | 177 | 0·29 |
| | 178 | 0·28 |
| | 181 | 0·26 |
| | 205 | 0·17 |
| *Helianthus annuus* | 172 | 0·52 |
| | 194 | 0·58 |
| | 195 | 0·57 |
| | 196 | 0·42 |
| *Cucurbita pepo* | 179 | 0·41 |
| | 187 | 0·37 |
| | 190 | 0·42 |
| *Ricinus communis* | 184 | 0·43 |
| | 185 | 0·40 |
| | 198 | 0·72 |

Turning now to a further consideration of the experiments with fat-storing seeds, it will be seen from Table IV that the NR/OR values for *Ricinus*, *Cucurbita*, and *Helianthus* are all greater than 0·33. It therefore may be possible that, in these, a certain amount of oxidative anabolism takes place.

One tentative suggestion that may be put forward as a partial explanation of the high values of NR/OR in fat-storing seeds is that oxygen supply may be a limiting factor which prevents the output of carbon dioxide in air reaching its full rate. We know from published chemical data (Pierce, Sheldon, and Murlin, 1933) that reserve fat in seeds is converted to sugar during germination, and that oxygen is absorbed and used in this conversion. In the germinating seeds of *Ricinus*, *Cucurbita*, and *Helianthus*, the low respiratory quotient values indicate that oxygen is being used in this way for the conversion of fat into sugar, as well as for the respiratory oxidation of the latter substance. Blackman (1930) has stated that in respiring apples carbon dioxide production " varies with every alteration of oxygen concentration in the environment". It may therefore be that, with these two processes going on in the same tissue and at the same time, the diffusion rate of oxygen, to the seats of the reactions involved, may be insufficient to allow the reactions to proceed at their maximum rates. This being so, the experimentally obtained values for the rates of carbon dioxide production in air would be something lower than three times those for the rates in nitrogen and thus NR/OR would have values greater than 0·33. A further reference to this point, however, will be made in a later paper of this series when some new experimental data which appear to throw light upon it are discussed.

The values of NR/OR obtained by extrapolation, at the moments when the gas surrounding the seedlings was changed from nitrogen to air, have been examined. Owing to the continued decrease in carbon dioxide production which always finally sets in when the seedlings are kept in nitrogen, and to the already mentioned accumulation of some substance which will readily combine with oxygen, these final NR/OR values show great variation in magnitude in different experiments. They therefore cannot be used for the purpose of confirming the above deductions obtained with the values given by the ratio at the transition from air to nitrogen.

Attention is now drawn to the rise which occurs in the anaerobic production of carbon dioxide, in carbohydrate seeds, immediately following the initial fall which takes place when the change from air to nitrogen is made. This rise is very definitely shown in the experiments with *Lathyrus* and *Zea* and is slightly indicated in those with *Fagopyrum*. Although chemical data are necessary for a final explanation of this rise, it suggests that in the absence of oxygen, some new process which involves the production of carbon dioxide, comes into operation and gradually assumes its maximum rate. It would appear that this process operates on a substrate which is only formed when oxygen is present and is thus, in absence of oxygen, limited in quantity. The activity of the process thus increases to a maximum, only to decrease again as the available supply of substrate is used up. This rise and fall in the rate of the process is indicated by the rise and fall in the carbon dioxide production which results from it.

The sequence of events which follows, when seedlings of *Lathyrus*, *Zea*, and *Fagopyrum* are transferred from air to nitrogen, may therefore be visualized very broadly as three phases which are outlined below.

I. The suspension of the oxygen supply brings about a change over from a complete oxidation of hexose to carbon dioxide and water, to its partial oxidation to carbon dioxide and alcohol. This change causes the carbon dioxide production in nitrogen to fall to one-third of what it was in air.

II. At the moment when the oxygen supply is cut off, the process, which has been discussed above, comes into operation. The activity of this process increases to a maximum and then decreases as its specific substrate diminishes in quantity. With this is associated an output of carbon dioxide which is independent of that referred to in connexion with phase I. This additional carbon dioxide production first rises and then falls as the reactions to which it owes origin, increase and then decrease in velocity.

III. As the respective substrates for both phases I and II are dependent on oxygen for their formation, they decrease in quantity in absence of oxygen. This decrease in substrate is indicated by the final decrease in anaerobic carbon dioxide output with time, which occurs when seedlings are kept in nitrogen for prolonged periods.

If the experimental data obtained with *Helianthus*, *Cucurbita*, and *Ricinus* are examined, evidence supporting the existence of phase II is not to be found. It may be that, in fat-storing seeds, oxygen supply during respiration in air, acts as a limiting factor on the production of substrate for this second anaerobic phase, in a similar way to that suggested as accounting for the high NR/OR values. The result being that the necessary substrate is absent or is only formed in quantities too small to give experimental evidence of its presence.

Chemical changes taking place in germinating seeds are being investigated in this department and until data regarding these are available the advisability of carrying the present discussion further is doubtful. It appears certain, however, that the respiratory processes of the actively growing tissues of germinating seeds differ in important details from those published by other workers for senescent tissues, such as occur in ripening apples as described by Blackman (1928) and in other fruits (Hill, 1913).

## SUMMARY

Experimental data are given relating to the changes that occur in carbon dioxide production and in the values of the respiratory quotient, when germinating seeds of *Lathyrus odoratus*, *Fagopyrum esculentum*, and *Zea mais* are kept successively in air, nitrogen, and air.

Data obtained are discussed in relation to the possible light they throw on the nature of the respiratory processes of germinating seeds.

## REFERENCES

Blackman, F. F. (1928). 'Proc. Roy. Soc.,' B, vol. **103**, p. 491.

—— (1930). "5th Int. Bot. Congr., Camb.," 'Rep. Proc. Publ. Cambridge,' 1931.

Hill, G. R. (1913). 'Bull. Cornell Univ. Agric. Exp. Sta.,' p. 330.

Jensen, P. Boysen (1923). 'K. danske Vidensk. Selsk. Skr.,' vol. **4**, p. 1.

Leach, W. (1932). 'Ann. Bot.,' vol. **46**, p. 583.

Leach, W., and Dent, K. W. (1934). 'Proc. Roy. Soc.,' B, vol. **116**, p. 150.

Pierce, H. B., Sheldon, D. E., and Murlin, J. R. (1933). 'J. gen. Physiol.,' vol. **17**, p. 311.

Stiles, W., and Leach, W. (1931). 'Ann. Bot.,' vol. **45**, p. 462.

—— (1933). 'Proc. Roy. Soc.,' B, vol. **113**, p. 406.

# INDEX TO VOLUME CXIX (B)

Accommodation, measurement in nerve (Solandt), 355.

Alcohol, coupled oxidation (Keilin and Hartree), 141.

Aniline, oxidation (Mann and Saunders), 47.

Anrep (G. V.), Pascual (W.), Rössler (R.) Respiratory variations of the heart rate. I—The reflex mechanism of the respiratory arrhythmia. II—The central mechanism of the respiratory arrhythmia and the inter-relations between the central and the reflex mechanisms, 191, 218.

Arrhythmia, respiratory, reflex mechanism (Anrep and others), 191, 218.

Bacterial cells, fluctuations in numbers in soil (Taylor), 269.

Barker (J.) Analytic studies in plant respiration. VI—The relation of the respiration of potatoes to the concentration of sugars and to the accumulation of a depressant at low temperatures. Part 3—The relation of the respiration to the concentration of sucrose, 453.

Broom (J. C.) *See* Brown and Broom.

Brown (H. C.) and Broom (J. C.) Studies in microcataphoresis. I—Technique, 231.

Butler (C. G.) and Innes (J. M.) A comparison of the rate of metabolic activity in the solitary and migratory phases of *Locusta migratoria*, 296.

Caldwell (J.) Factors affecting the formation of local lesions by tobacco mosaic virus, 493.

Courtice (F. C.) and Douglas (C. G.) The effects of prolonged muscular exercise on the metabolism, 381.

Cyanide, action on xanthine oxidase (Dixon and Keilin), 159.

Dixon (M.) and Keilin (D.) The action of cyanide and other respiratory inhibitors on xanthine oxidase, 159.

Douglas (C. G.) *See* Courtice and Douglas.

Food-habit, induced, transference from parent to offspring, II (Sladden), 31.

Hartree (E. F.) *See* Keilin and Hartree.

Heart rate, respiratory variations, I and II (Anrep and others), 191, 218.

Hill (A. V.) Excitation and accommodation in nerve, 305.

Hill (A. V.) The strength-duration relation for electric excitation of medullated nerve, 440.

Innes (J. M.) *See* Butler and Innes.

Keilin (D.) *See* Dixon and Keilin.

Keilin (D.) and Hartree (E. F.) Coupled oxidation of alcohol, 141.

Keilin (D.) and Hartree (E. F.) Uricase, amino acid oxidase, and xanthine oxidase, 114.

Leach (W.) Researches on plant respiration. IV—The relation between the respiration in air and in nitrogen of certain seeds during germination. (*b*) Seeds in which carbohydrates constitute the chief food reserve, 507.

*Locusta migratoria*, comparison of the rate of metabolic activity in the solitary and migratory phases (Butler and Innes), 296.

*Locusta migratoria* L, effects of X-rays on mitosis in the spermatogonial divisions (White), 61.

Mann (P. J. G.) and Saunders (B. C.) Studies in peroxidase action. I—The oxidation of aniline, 47.
McCance (R. A.) Experimental sodium chloride deficiency in man, 245.
McKeown (T.) *See* Selye and McKeown.
Metabolism, effects of prolonged muscular exercise (Courtice and Douglas), 381.
Microcataphoresis, studies, I (Brown and Broom), 231.
Mitosis, effects of X-rays in the spermatogonial divisions of *Locusta migratoria* L (White), 61.

Nerve, excitation and accommodation (Hill), 305.
Nerve, measurement of " accommodation " (Solandt), 355.
Nerve, medullated, strength-duration relation for electric excitation (Hill), 440.

Oxidase, amino acid, and xanthine (Keilin and Hartree), 114.

Pascual (W.) *See* Anrep and others.
Peroxidase action, I (Mann and Saunders), 47.
Placenta, maternal, studies on physiology in rat (Selye and McKeown), 1.
Plant respiration, VI (Barker), 453.
Plant respiration, researches, IV (Leach), 507.
Potatoes, relation of respiration to concentration of sugars (Barker), 453.

Respiration, plant (Barker) 453; (Leach), 507.
Richardson (G. M.) The principle of formaldehyde, alcohol, and acetone titrations, 85.
Rössler (R.) *See* Anrep and others.

Saunders (B. C.) *See* Mann and Saunders.
Selye (H.) and McKeown (T.) Studies on the physiology of the maternal placenta in the rat, 1.
Sladden (D. E.) Transference of induced food-habit from parent to offspring, II, 31.
Sodium chloride deficiency in man (McCance), 245.
Soil, short-period fluctuations in the numbers of bacterial cells (Taylor), 269.
Solandt (D. Y.) The measurement of " accommodation " in nerve, 355.

Taylor (C. B.) Short-period fluctuations in the numbers of bacterial cells in soil, 269.
Thornton (H. G.) The action of sodium nitrate upon the infection of lucerne root-hairs by nodule bacteria, 474.
Titrations, principle of formaldehyde, alcohol, and acetone (Richardson), 85.

Uricase, amino acid, oxidase, and xanthine oxidase (Keilin and Hartree), 114.

Virus, tobacco mosaic, factors affecting the formation of local lesions (Caldwell), 493.

White (M. J. D.) The effects of X-rays on mitosis in the spermatogonial divisions of *Locusta migratoria* L, 61.

Xanthine oxidase, action of cyanide and other respiratory inhibitors (Dixon and Keilin), 159.

END OF THE ONE HUNDRED AND NINETEENTH VOLUME (SERIES B).

I. A. R. I. 75.

**IMPERIAL AGRICULTURAL RESEARCH INSTITUTE LIBRARY**
**NEW DELHI.**

| Date of issue. | Date of issue. | Date of issue. |
|---|---|---|
| 10.12.57 | | |
| 13.2.59 | | |
| 26.2.64 | | |
| 8.2.65 | | |
| | | |
| 1-2-66 | | |
| 9.9-66 | | |
| 29.8.67 | | |
| -2 JAN 1971 | | |
| | | |
| | | |
| | | |
| | | |
| | | |